AN HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF RESIDUAL BENOMYL AND MBC ON APPLE FOLIAGE WITHOUT CLEANUP

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ABSTRACT

A simple High Performance Liquid Chromatograph (HPLC) method has been developed to identify benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate) and MBC (methyl 2-benzimidazole carbamate) residues on apple leaves without cleanup. Sample leaves are freeze dried in a Mason jar and residues are then extracted by tumbling them in chloroform containing 5,000 microgram per milliliter of n-propyl isocyanate (PIC) at 1°C. To the extract, n-butyl isocyanate (BIC) was added at 5,000 microgram per milliliter and 20 microliter of this mixture injected onto the HPLC system. Separation is accomplished by the use of a Brownlee LiChrosorb silica gel column with a guard column and operated with a mixed mobile phase consisting of chloroform and hexane (4:1) saturated with water. MBC, a degradation product of benomyl is identified if present as methyl 1-(n-propyl carbamoyl)-2-benzimidazole carbamate (MBC-n-PIC). Both benomyl and MBC-n-PIC can be detected with an Ultraviolet (UV) detector (280nm) at a concentration as low as 0.2 microgram per milliliter in apple leaves. The fate of benomyl on apple foliage after spray application of benomyl (Benlate 50 per cent wettable powder) was investigated by the method thus described. Benomyl quickly dissipated during the first 3-7 days, but the dissipation slowed down thereafter. In contrast, the concentration of MBC in leaves gradually increased after repeated applications of Benlate.

ACKNOWLEDGEMENTS

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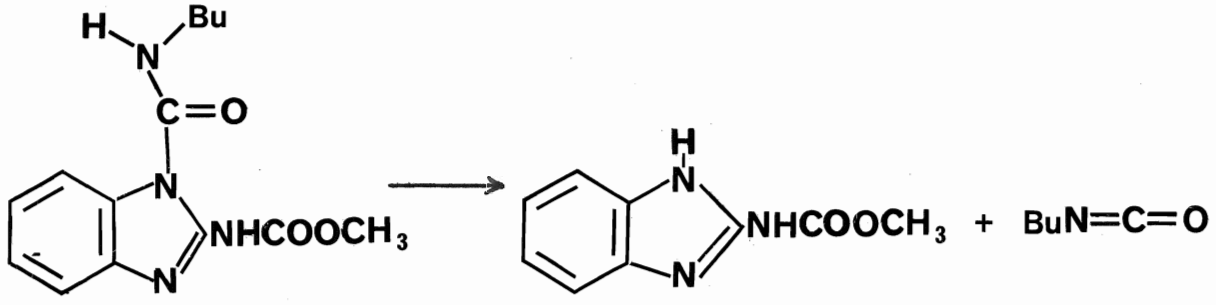
INTRODUCTION-THE BENOMYL ANALYSIS PROBLEM

Benomyl (trade name Benlate^{R}) is a systemic fungicide manufactured by DuPont. Benomyl (I), methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate decomposes in organic solvents to methyl 2-benzimidazole carbamate (MBC) (II), and n-butyl isocyanate (III). The kinetics of the decomposition of benomyl in organic solvents has been extensively



(I) (II) (III)

studied by Calmon and Sayag (1) and by Chiba and Cherniak (2). Chiba and Cherniak determined the rate constants and equilibrium constants for benomyl in chloroform, benzene, methanol, ethanol, ethyl acetate, methylene chloride and p-dioxane. The significance of these data is that benomyl decomposes at different rates depending on the solvent. The amount of benomyl remaining at equilibrium at a fixed starting concentration is also determined by the solvent. Of the solvents investigated by Chiba and Cherniak (2) benomyl in chloroform exhibited the slowest decomposition rate at 25^{o}C and also

had one of the highest concentrations of benomyl remaining at equilibrium. Only benzene had a higher concentration of benomyl remaining at equilibrium. Because of benomyl's decomposition in organic solvents this meant that while benomyl was being extracted from whatever substrate, or being cleaned-up for analysis it was continuously decomposing to MBC and n-butyl isocyanate (BIC). It wasn't until a spectrophotometric method was developed by Chiba (3) that benomyl intact and MBC could be quantitatively determined. Unfortunately the aforementioned method was only good for pure compositions of benomyl and MBC because any ultraviolet (UV) absorbing coextractives present would make the determination of benomyl and MBC present impossible. This method for the spectrophotometric determination of benomyl and MBC was modified by changing the solvent to a 3 component mixture enabling the analysis of wettable powder (WP) formulations (4). Extraction of benomyl at low temperature to minimize its degradation in solvents is an essential part of this procedure to determine benomyl intact. Unfortunately again this method is not suitable for the determination of residual benomyl in agricultural crops because of the interference from coextractives.

Several methods have been published to date that describe a method of analysis for benomyl and /or MBC. Paolo Cabras and co-workers developed a method for the analysis of benomyl and MBC by reverse phase chromatography (5). The

authors used a mobile phase of acetonitrile and water. They extracted benomyl and MBC from grapes using petroleum ether. The organic layer was dried using sodium sulfate, evaporated and re-dissolved into acetonitrile. The separation of MBC from the void volume is poor and no mention throughout the paper is made regarding benomyl degradation. During the extraction procedure, the evaporation and actual chromatogram run, no precautions were taken to prevent benomyl degradation.

Kirkland and co-workers developed an HPLC method for benomyl and MBC residues (6). Any residues are extracted by refluxing for several hours in acidic methanol. The methanol extract is concentrated and a sodium hydroxide solution added to the concentrate. The extract is cleaned up by liquid-liquid partitioning with chloroform after which the chloroform extract is discarded. The MBC is then extracted from the aqueous phase using ethyl acetate. The ethyl acetate is evaporated and the residues re-dissolved into a 0.1 N H_3PO_4 solution. The analysis is carried out using a high speed cation exchange column and a UV detector. Recoveries from such samples as peaches, apricots, cherries, grapes, orange peel, orange pulp, celery, pecans, apples, cucumbers, cantalope, squash, corn kernel,corn foliage, carrot roots, snap beans, sugar beet roots and sugar beet foliage are documented. The average recoveries for these samples ranged from a low of 67 per cent to a

high of 100 per cent. The major drawback of this method is that benomyl is not determined as benomyl but rather degraded and then measured as MBC. Essentially the same method of benomyl is used by Farrow and co-workers (7). In the Farrow paper they concentrated on the analysis of citrus fruit and unlike Kirkland used normal phase, reverse phase and cyano bonded columns for the analysis.

A spectrophotometric method for the analysis of benomyl by Miller and co-workers is able to measure benomyl in the 200 microgram to 12 milligram range (8). This is done by complexing benomyl with copper and measuring the colored complex at 420 nanometers. The authors used this method to measure the benomyl concentration in treatment tanks. Rouchaud and Decallonne analyzed benomyl by degrading it to MBC, derivatizing the MBC to a trifluoroacetyl derivative and analyzing by gas chromatography with an electron capture detector (9). The stated sensitivity was as low as 0.02 parts per million.

An analytical method that can simultaneously determine benomyl and MBC while preserving the integrity of the original benomyl present is needed. Such a method would be useful in determining the mode of action of benomyl and MBC since both are fungitoxic. The rate of degradation of benomyl to MBC after spray application could be determined providing information on benomyl's persistance in the field. The analysis of benomyl residues on different food crops

could also be determined.

A method for the simultaneous determination of benomyl and MBC on apple foliage after spray application is described herein. The method is carried out using a HPLC with a fixed wavelength UV detector. MBC is difficult to extract with chloroform (benomyl is extremely soluble in chloroform) and because of its different eluting characteristics in HPLC systems, it was derivatized to 1-(n-propyl carbamoyl)-2-benzimidazole carbamate (MBC-n-PIC). The derivatization takes place by reacting MBC with n-propyl isocyanate (n-PIC). The MBC present in the sample is derivatized during the extraction to MBC-n-PIC and determined simultaneously with benomyl under one set of HPLC conditions.

INTRODUCTION-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Almost all sample analysis requires the separation of the required analyte from a complex matrix of interfering components. To accomplish this, a variation of chromatography is used. Chromatography is a physical method of separation in which a complex mixture is separated by distributing it between two phases, a mobile phase and a stationary phase. The solute partitions itself between the two phases depending on its affinity for the two phases. If the solute has a stronger affinity for the stationary phase it will be retained and have a long retention time (t_r). However if the affinity of the solute is stronger for the mobile phase the solute will be carried quickly through the column and have a short retention time. A characteristic band width (t_w) is associated with every eluting peak. The band width is measured by drawing tangents to each side of the peak down to the baseline. The distance between the two tangents that touch the baseline is the bandwidth. The narrower the bandwidth the better the resolution capability of the column. A common way of expressing the efficiency is to use the column theoretical plate number:

$$N=16\left(\frac{t_r}{t_w}\right)^2$$

By using the equation a measure of the columns efficiency can be made. From the equation when the value of t_w is small

greater efficiency occurs. Also for a constant plate count the equation predicts the longer the retention time on the column the wider the bandwidth. Experimentally this is the case as the solute stays on the column its bandwidth will be proportional to its retention time. As the retention time increases at some time "t" the peak will disappear into the baseline. The length of the column also plays an important part in the plates of the column. As the column length increases the plates will proportionally increase as shown below:

$$N=\frac{L}{H}$$

In the equation N is the total number of plates, the length of the column is L and H, is the height equivalent to a theoretical plate (HETP). Obviously the smaller the value of H the more plates, the higher the efficiency.

The fundamental concept of chromatographic separations can be described by using the gas chromatographic Van Deemter equation. The Van Deemter equation describes the relationship between the height equivalent of theoratical plate and the mobile phase linear velocity.

$$H = A + \frac{B}{\mu} + C\mu \quad (10)$$

The A term describes the dispersion from the spaces between the particles and how well the column is packed. The B term which reflects the longitudiual diffusion of a liquid is negligible and is of little consequence to the equation. The C term is used to describe the nonequilibrium that results from resistance to mass transfer in the stationary and mobile phases of the column. The characteristics of the packing material and also its relationship with eluant and solute, control the C term.

Several factors can be used to optimize H and obtain the maximum number of theoretical plates. H is small for small diameter column packing, for example 5, 10 or 20 micron packings. By using optimum flow rates, typically 0.8 milliliter per minute yields a small value of H. H can also be optimized using less viscous solvents and operating at high temperatures.

Another important factor in HPLC to consider is resolution. Resolution is a measure of how much two components are separated. It can be defined as the difference in retention times divided by the mean peak (base) width.

$$R_s = \frac{t_R^A - t_R^B}{1/2\ (W_A + W_B)} \quad (10)$$

If the R_s value is 0.8 or less there is only partial resolution and some overlap between the peaks. A value

of 1 shows a small overlap and a value 2 shows excellent separation with no overlap unless the peaks tail.

A basic HPLC unit will contain four main components which are, a pump, an injection system, a column and a detector.

Although there are many different types of pumps the most commonly used one is called a reciprocating piston pump. With this pump a piston is driven mechanically with motors and gears or by solid-state pulsing circuits. In both of these cases the solvent is pulled in from the reservoir on the intake stroke and then on the return stroke the reservoir valve is closed and the solvent forced onto the column. This type of pump will produce pulsations which can be reduced either by using electronic sensors to sense the pressure between pump strokes and then speed up or slow down the motors to minimize pulsations.

Two types of injection systems are most commonly used, they are the syringe injection and the sample valve systems. In the syringe injection system the sample is loaded into a syringe which passes the sample via a needle through a septum and into a loading loop. Samples from a microliter to a milliliter can be injected into the loading loop. Once the sample is in the loop the valve is turned and the loop is placed in the solvent stream before the column. The other injection system, the sample valve design has the

sample filling the sample loop which is of a fixed known volume. By then turning the valve the loop is placed in the solvent stream before the column. This method is the most convenient and reproducible method of sample injection and sample size can be easily changed by changing the sample loop.

The column if referred to as the "heart" of an HPLC system and it is responsible for the main function of a HPLC, that is to separate components in complex mixtures. The size of the column packing material varies, but the most commonly used are the 5 and 10 micron, particle sizes. Four basic types of packings are available today which are normal phase, reverse phase, ion exchange and gel permeation packings. Normal phase packings include silica gel and alumina type materials which can be used with non polar solvents such as chloroform, hexane, pentane and others. Reverse phase packings consist of a silica gel backbone with hydrophobic chains bonded to it. The most common reverse phase columns have carbon chain lengths of C_2, C_8, and C_{18} bonded to the silica gel backbone. Ninety per cent of all reverse phase applications can be handled by using a polar solvent mobile phase of either water-methanol or water-acetonitrile (11). Most ion-exchange packings consist of crosslinked porous resins and are available in both anion and cation exchange columns. Solvents used for anion and cation exchange columns include water-methanol, acids

(acetic,phosphoric) and numerous buffer systems. Gel permeation columns consist of two types, which are rigid and semi-rigid packings. The rigid packings include controlled size glasses and silicas which are both compatible with all solvents. The semi-rigid packings consist of polystyrene gel which is compatible with all solvents except alcohols and water.

Detectors used for HPLC are numerous, but the most commonly used ones are Ultra-violet and visible (UV-VIS) variable and fixed wavelength, refractive index (RI) and fluorescence detectors. When comparing minimum concentration detectable the fluorescence is the best at 10^{-12} gram per milliliter followed by 10^{-9} gram per milliliter for fixed wavelength UV-VIS, 4×10^{-9} gram per milliliter for variable wavelength UV-VIS and 7×10^{-7} gram per milliliter for refractive index detectors (12). The electrochemical detector is gaining wider acceptance but is at a slight disadvantage because it is restricted to aqueous mobile phases compatible with reverse phase and ion exchange columns. Some of the more infrequently used more sophisticated detectors include electron capture, mass spectrometry, plasma chromatography, transport flame ionization, atomic absorption, flame emission, photoionization and flame photometric (12). In addition some reaction detectors have been developed such as the cerium oxidative, nitroso-compound and fluorometric enzyme inhibitor detectors.

These detectors are specific for the detection of oxidizable compounds, nitrosamides and carbamates respectively (13).

The future of detectors for HPLC looks bright with the possibility of interfacing some unusual detectors in the very near future. The possibility exists for the interfacing of HPLC with such systems as gas chromatography, Raman spectroscopy, nuclear magnetic resonance and microwaves (14). This will further allow the analyst to separate components from complex mixtures and conclusively identify them.

Many other accessories are available for use with the HPLC that enhance its versatility. Programmable variable wavelength detectors are available that make it possible to look at eluting components, each at their own max. The increase in sensitivity using this technique can be quite substantial. Autosamplers are useful in that they allow the analyst to load samples and standards for overnight unattended analysis. When the autosampler is combined with microprocessor it is possible to program each indiviual sample for run time, injection volume, number of injections per sample vial, and equlibrium delay (time necessary for the column to return to initial conditions after a gradient run). The system microprocessor is capable of controlling flow rate, oven temperature, solvent composition (mobile phase), pressure limits (upper and lower) which protects against a plugged filter or a leak in the system. The microprocessor is capable of controlling external devices such

as solenoid valves used for changing solvents or for changing columns. The coupling of an integrator with a printer plotter make a powerful combination. With this combination it is possible to plot out spectra, label peaks for retention time and calculate peak areas for quantitation. The use of magnetic tapes make it possible to store all the working details of an analytical method. With the entire method on tape it is possible to call the method out of memory into active use or to edit the method. The HPLC can be coupled to a stand alone computer where data can be stored permanently or temporarily to be manipulated at the operators convenience.

When a solute is to be chromatographed for the first time in HPLC method development a standard approach is taken. For example in reverse phase the approach is to start with a mobile phase of 100 per cent methanol (a strong solvent for reverse phase chromatography) and slowly decrease the methanol % of the mobile phase until either separation is achieved or 100 per cent water is reached. When using normal phase the usual starting solvent is chloroform which should elute the component easily from the column. The concentration of hexane in chloroform is increased until the separation is achieved or the mobile phase is 100 per cent hexane. Other solvents can also be used in both cases. For example in reverse phase acetonitrile is very often used in place of methanol. For normal phase systems, other solvents

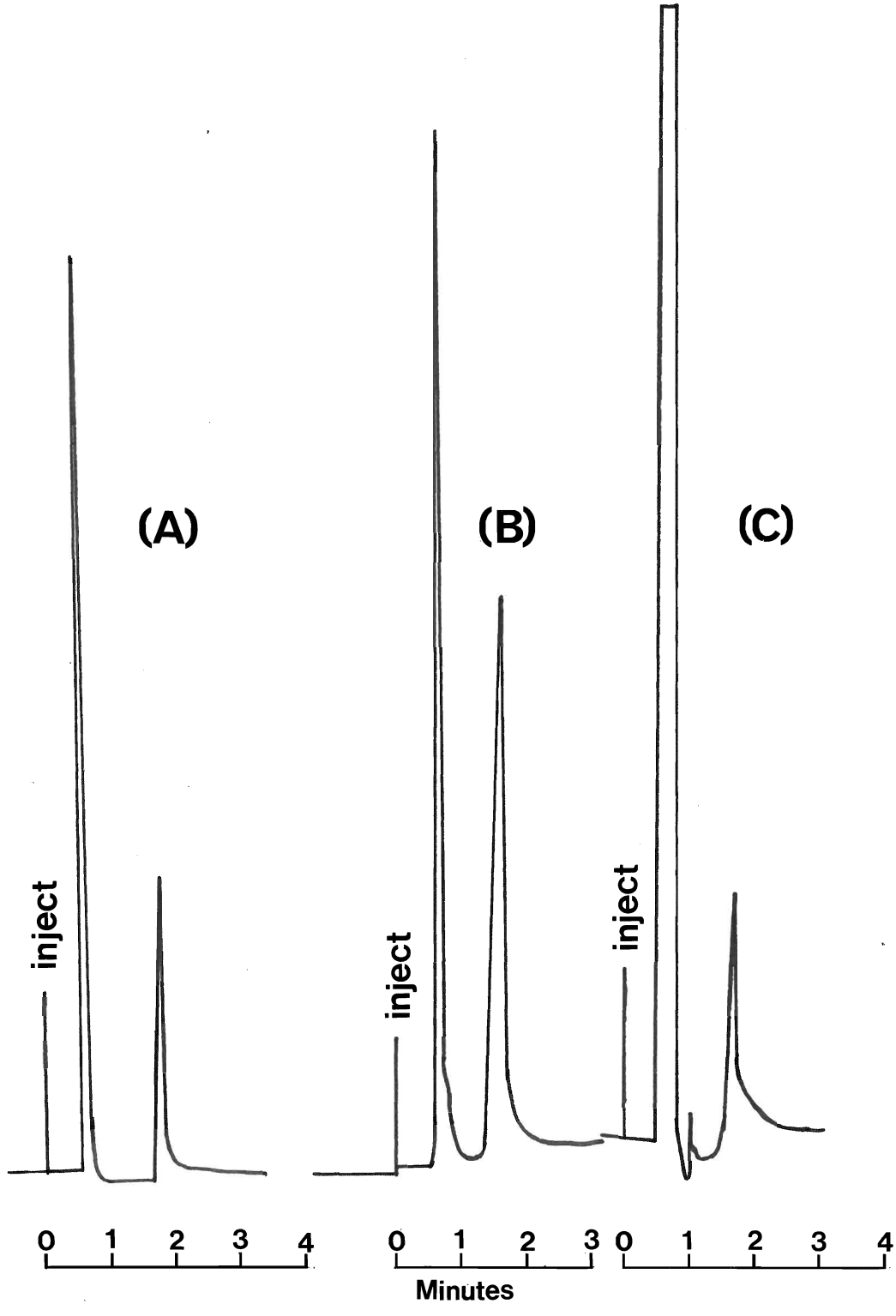
used in place of chloroform are methylene chloride, ethyl acetate, tetrahydrofuran and ethanol to mention a few.

PRELIMINARY STUDY

The analysis first attempted was the simultaneous determination of benomyl and MBC in the apple leaf extract. In the beginning it was felt that it may be possible to separate benomyl and MBC at the same time from the apple leaf extract background without cleanup prior to injection. This initial work was done using a Corasil II (Waters Scientific) 37-50 micron particle size, 2 mm I.D. X 61 cm column. Corasil II is a silica gel pellicular packing and therefore has a small surface area. This particular column had 1,000 plates per the 61 cm length of the column. Since the solubility difference between benomyl and MBC was known the extracting solvent always contained a mixture of chloroform (for benomyl) and p-dioxane (for MBC) in hexane. The extractions of the blank leaf sample were always carried out at 1^{o}C to simulate the conditions that would be necessary to extract a real sample (conditions that would be necessary to prevent the decomposition of benomyl during the extraction step). A series of mobile phase compositions were tried in an attempt to achieve benomyl and MBC separation from the background peaks of the apple leaf extract. Mobile phase compositions ranging from 5 per cent chloroform in hexane to 100 per cent chloroform were tried with none of the mixtures giving a satisfactory separation. The apple leaf extract background would always interfere with the elution area of benomyl. Figure 1 illustrates the typical

Figure 1

Typical Chromatograms using the Corasil II column



elution patterns obtained for MBC, benomyl and the apple leaf extract. Figure 1A is the elution for MBC, figure 1B for benomyl and figure 1C for the apple leaf extract using a mobile phase of 50 per cent chloroform in hexane. In this example the elution of benomyl is interfered with by the elution of the apple leaf extract component at the same retention time. The conclusion was reached that this column with its 1,000 plates would never be able to carry out the separation from the apple leaf extract. A column with more theoretical plates or a different selectivity had to be tried.

The next column used was a micro Bondapak cyano (Waters Scientific) column, which is a cyano bonded silica backbone of intermediate polarity. This column contained a 10 micron packing material and had dimensions of 3.9 mm I.D. X 30 cm. The theoretical plate count was in the range of 4,000 plates per 30 cm of column length. This represents a considerable increase in plates over the Corasil II column. The apple leaf extract no longer eluted as a single peak but eluted as several peaks. Benomyl could be much more efficiently separated from MBC and the main apple leaf extract peak. Several mobile phase compositions were tried including 100 per cent of chloroform, p-dioxane and methanol. Combinations of water-methanol and water-p-dioxane were also tried but no combination of solvents provided the separation needed. Some of the typical chromatograms

are shown in Figure 2 and Figure 3. The mobile phase for this series of chromatograms is 50/50 p-dioxane and water. Figure 2A illustrates the elution of benomyl. Figure 3 shows the elution obtained for the apple leaf extract, with the point to note being the interference of the apple leaf extract at the elution times of benomyl and MBC.

The next attempt at analyzing benomyl and MBC involved using 2 columns. The first column contained a gel permeation packing (GPC) called Fractogel PVA-500 (manufactured by E. Merck, Darmstadt, Germany). It was possible to separate MBC from the apple leaf extract on this column using a solvent that could also achieve separation for benomyl from the apple leaf extract on the second column. The benomyl eluted with the apple leaf extract from the GPC column onto a silica gel (10 micron, 4.6 mm X 25 cm, silica gel column manufactured by Brownlee Labs, Santa Clara California) column. Once the apple leaf extract and benomyl were eluted from the GPC column onto the silica gel column (Figure 4A) the valve is switched to remove the silica gel column from the solvent flow (Figure 4B). With the valve now switched MBC can elute from the GPC column and be quantitated. Once all the MBC has passed through the GPC column and been quanitated the valve is switched back (Figure 4A) and now benomyl can be separated from the apple leaf background and the benomyl quantitated.

The big problem with this system was that it required

Figure 2

Typical Chromatograms using the Cyano Bonded Phase Column

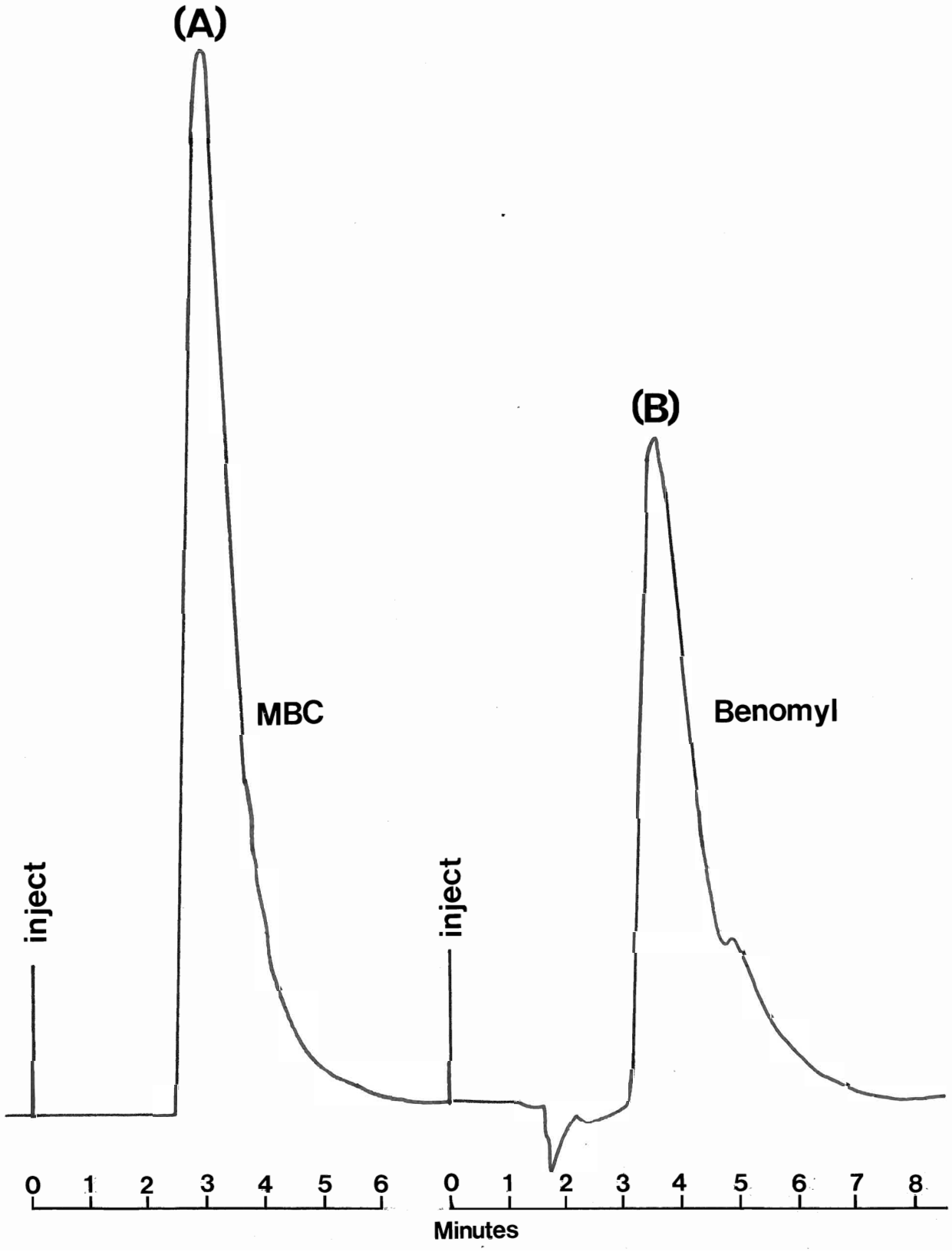


Figure 3

Typical Chromatogram using the Cyano Bonded Phase Column

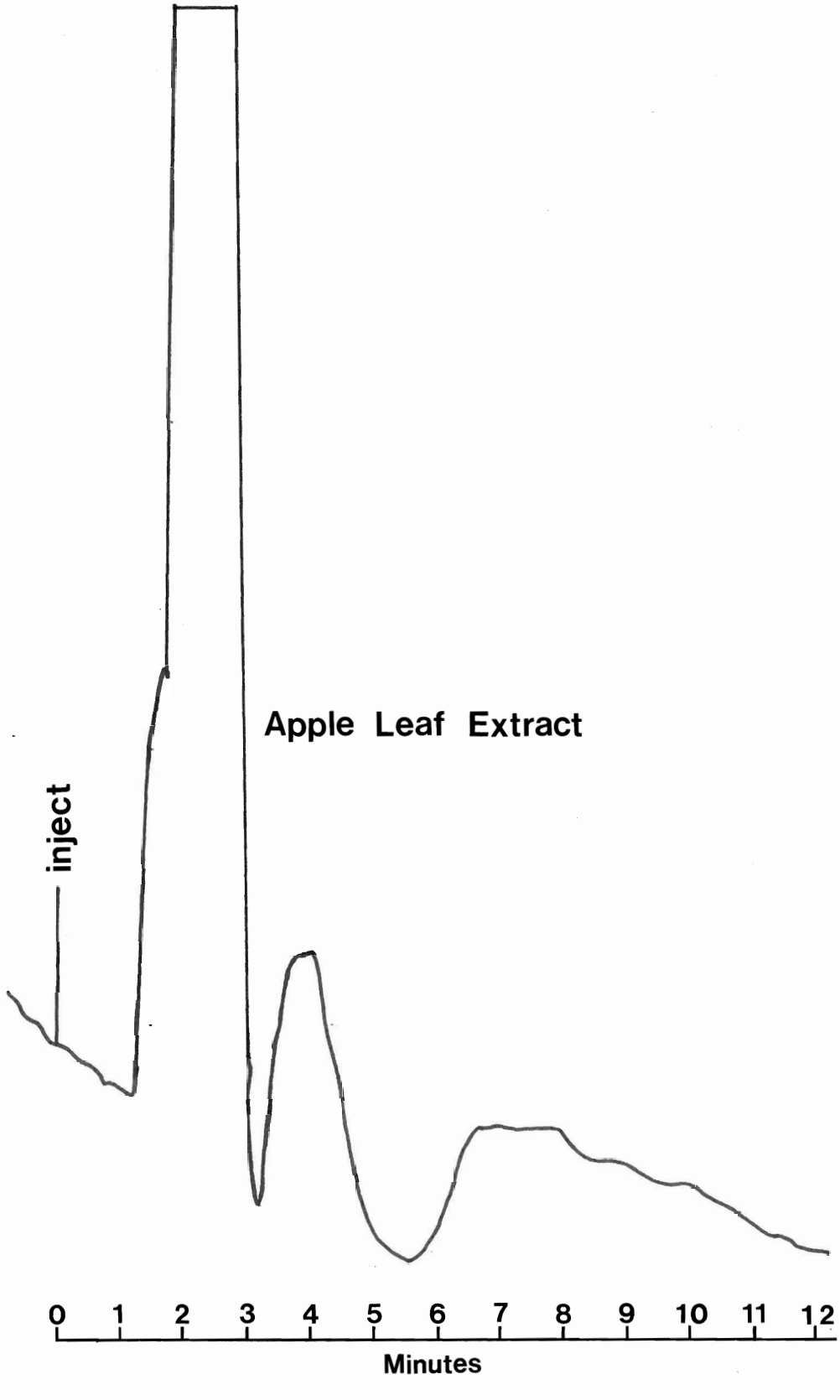
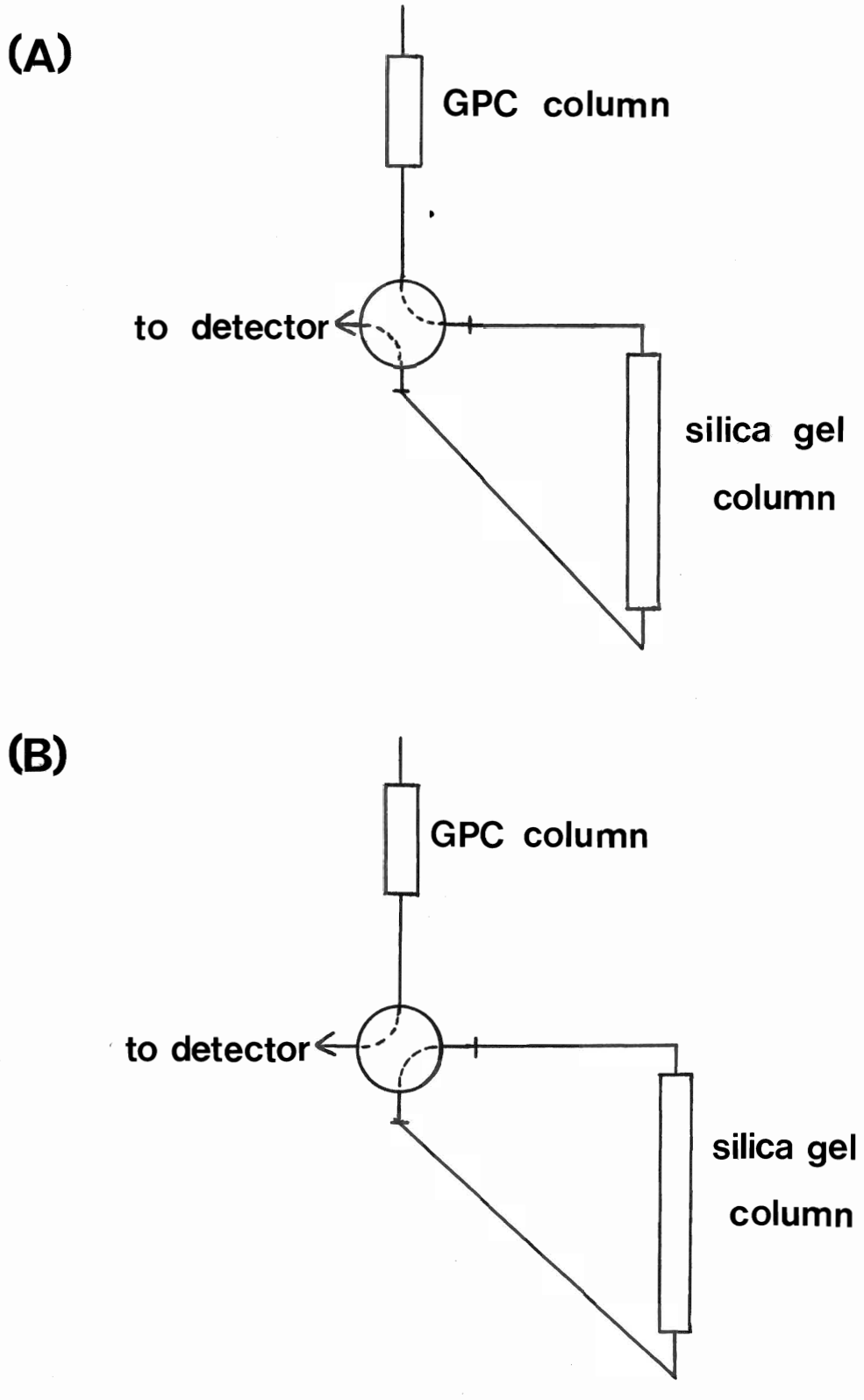


Figure 4

Switching Valve Diagram for the Analysis of MBC and Benomyl

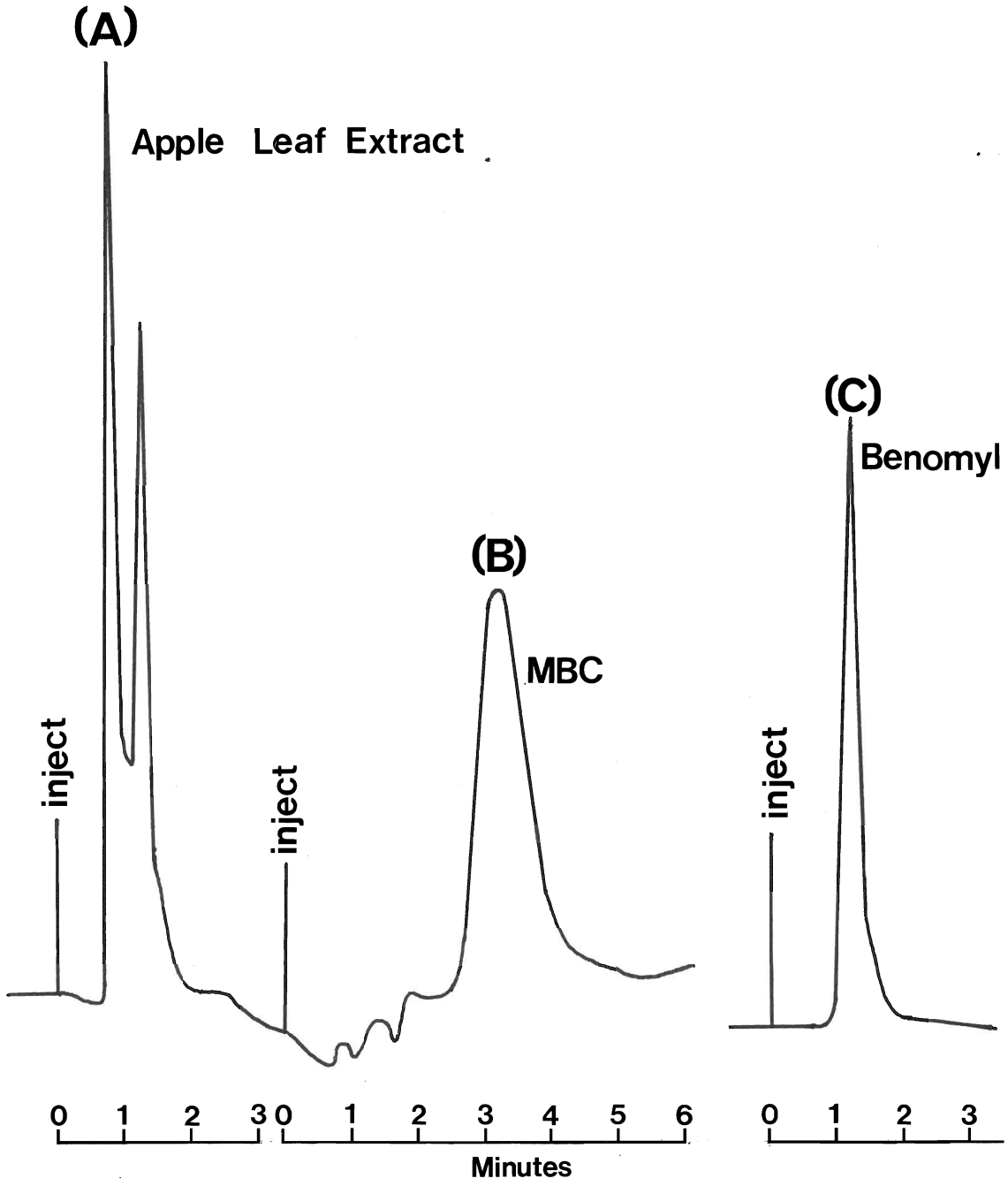


too much time. While the MBC was being quantitated on the GPC column the benomyl had to sit at the top of the silica gel column. In this period of time benomyl would be decomposing and so intact benomyl would be impossible to determine. In order for this method to be reproducible, the switching valve had to be switched at very precise times. Ideally this would and should be done by a microprocessor controlled pneumatic switching valve that was unavailable at the time of this method development. A typical chromatogram of the elution of the apple leaf extract, MBC and benomyl on the GPC PVA-500 column are shown in Figure 5.

The last solvent system tried was a mixture of hexane and chloroform with a normal phase silica gel column. At first 100 per cent chloroform was tried but benomyl eluted too quickly. As a result hexane was added to slow down the elution of benomyl from the column. A mixture of 80 per cent chloroform and 20 per cent hexane worked well. However the chloroform removed the moisture from the silica gel column and the benomyl retention time increased with each injection. In order to control the activity of the silica gel the mobile phase had to contain a fixed volume of water. This was accomplished by saturating the mobile phase with water. Under these conditions reproducibile retention times for benomyl were obtained.

Figure 5

Typical Chromatograms using the GPC PVA-500 Column



EXPERIMENTAL

(I) Materials and Reagents

(1) Benomyl - Benlate (Benlate is a registered trademark of DuPont) 50 per cent WP was used to prepare analytical grade benomyl. Benlate 50 per cent WP was dissolved into chloroform (no ethanol preservative) containing 10,000 microgram per milliliter BIC to preserve the benomyl intact. Hexane was added to the chloroform solution until it just turned slightly cloudy. The solution was allowed to stand for several hours until the majority of the benomyl had crystallized out. The resulting crystals were recovered using a buchner funnel and the crystals then freeze dried. This procedure was repeated until the resulting benomyl crystals reached a purity of 99.5 per cent or greater when compared against analytical grade benomyl supplied by DuPont. The purity of benomyl was determined by measuring the peak height at 294 nm of both the prepared standard and the DuPont standard at a concentration of 10 microgram per milliliter each.

(2) MBC - Some of the benomyl prepared above was placed in methanol and refluxed for several hours. The MBC thus formed was removed from the reflux apparatus and the methanol removed by gently heating. The resulting residue was redissolved into p-dioxane by gently heating. Once all the MBC dissolved hexane was added until the solution just started to turn cloudy. The solution was left for several

hours and the MBC crystallized out. The purity of the MBC was determined by the method developed by Chiba (3).

(3) MBC-n-PIC derivative - A MBC saturated solution in chloroform (no ethanol preservative) was prepared. To nine portions of the saturated MBC solution, one portion of a solution containing 100,000 microgram per milliliter of n-propyl isocyanate in chloroform was added. This solution was well mixed and after an hour of sitting hexane was added to precipate the MBC-n-PIC out of solution. The resulting MBC-n-PIC was re-dissolved into chloroform containing 10,000 microgram per milliliter of PIC and recrystallized by the same method used for benomyl above. If a known concentration of MBC-n-PIC derivative prepared had the same absorbance as an equivalent concentration of the MBC-n-PIC derivative prepared from a known concentration of MBC (analytical grade) dissolved in 10,000 microgram per milliliter of PIC in chloroform, then the derivative prepared was sufficiently pure. If the MBC-n-PIC derivative prepared was not sufficiently pure then more recrystallizations were carried out.

(4) Isocyanates - n-butyl isocyanate and n-hexyl isocyanate were obtained from Eastman Kodak Co., Rochester, N.Y.,14650. Ethyl isocyanate, methyl isocyanate and n-propyl isocyanate were obtained from Aldrich Chemical Co., Milwaukee, WI., 53201. All the above mentioned isocyanates are strong lachrymators

(5) Solvents - Both chloroform with and chloroform without ethanol were used. The chloroform with 1 per cent $^{v}/_{v}$ ethanol was used to prepare the HPLC mobile phase and chloroform with no ethanol preservative was used for extracting the sample. Both of the chloroforms used and also hexane UV grade were obtained from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Hexane spectrograde was also obtained from Caledon Laboratories Ltd., Georgetown, Ont. Distilled water was prepared at Vineland Research Station.

(6) Extracting solvent - A PIC solution at 5,000 microgram per milliliter in chloroform (without ethanol preservative) was used as the extracting solvent. The prepared solvent was kept at 1°C before and during use.

(7) Standard solutions - A BIC in chloroform solution was prepared at a concentration of 10,000 microgram per milliliter. This solution was used to dissolve all the benomyl standards. The standard solutions of benomyl were prepared in the range of 0.1 - 250 microgram per milliliter. A PIC in chloroform solution was prepared at a concentration of 10,000 microgram per milliliter. This solution was used to dissolve all the MBC-n-PIC standards which were prepared in the range of 0.1 - 1,000 microgram per milliliter. Both standards were prepared using the chloroform with no ethanol preservative.

(8) HPLC mobile phase - A mixed solvent consisting of chloroform (with 1 per cent $^{v}/_{v}$ ethanol preservative) hexane

and water was prepared as follows: mix well 80 milliliter of chloroform and 20 milliliter of hexane in a 250 milliliter glass separatory funnel. To the mixture 100 milliliter of distilled water were added and the mixture shaken well. After 10 min. the separation was complete and the bottom layer was drawn off and used as the mobile phase. The reproducibility of the HPLC results depends on the consistency with which the mibile phase can be prepared. A detailed study of the mobile phase is described later herein (Section V).

(9) Samples - Apple leaves (McIntosh and Delicious) sprayed with Benlate 50 per cent WP at 1.7 kilogram per hectare.

(II) APPARATUS

(1) Freeze dry apparatus - Virtis freeze drier, Gardiner, New York, Model # 10-146.

(2) Tumbler for extraction - Fisher-Kendall Mixer, Fisher Scientific Company, Don Mills, Ont.

(3) High Performance Liquid Chromatograph - assembled at the Research Station, Agriculture Canada, Vineland Station, Ont. Major components of the system are:

(A) Pump - Milton Roy, Mini-pump, 5,000 psi.

(B) Detector - Spectra-Physics model SP8200 UV-VIS fixed wavelength detector.

(C) Injector - Rheodyne Model 70-10 injector, sample loop 20 microliter.

(D) Column - Brownlee LiChrosorb Silica gel (10 micron Si-100 angstrom) column, 4.6 mm I.D. X 25 cm with a guard column, 4.6 mm I.D. X 3 cm, packed with 10 micron silica gel.

(E) Millipore filter - Type LC 10.0 micron filter with 25 mm stainless steel fitting.

(F) Coldspot freezer - Model RSP-9, inside dimension: 50 X 90 X 50 (high) cm

(III) SAMPLE PREPARATION AND EXTRACTION

(1) Sample preparation - a total of 50.0 gram of apple leaves were placed (with stem removed and kept frozen at -15^{o}C, 1-2 mm Hg pressure for 2 hours.

(2) Extraction - The freeze dried leaves were kept in the freezer along with the tumbler. To the leaves 100 milliliter of PIC containing chloroform (maintained at $1^{o}C$) was added and the leaves were tumbled for 10 min. at $1^{o}C$ in the freezer. The extracting solvent was decanted into a 250 milliliter volumetric flask and 1.25 gram of BIC added. The extraction was repeated again with 100 and then 50 milliliter of the extracting solvent, extracting for 10 min. each respectively. Both extracts were decanted to the 250 milliliter volumetric flask via a glass funnel. The volumetric flask was made to volume with the extracting solvent. The extraction of the leaves, the storage of the extracting solvent and the storage of the extracted leaves were kept at $1^{o}C$.

(3) Filtration - 2 milliliter of the resulting solution from the volumetric was passed through a 10 micron millipore filter. The filtrate was then used for injection on the column.

(IV) HPLC ANALYSIS PARAMETERS

The mobile phase was run isocratically at a flow rate of 1 milliliter per min. The column was maintained at room temperature. The SP8200 detector (with appropiate lamp and filter) was operated at 280 nm. A volume of 20 microliter (fixed loop) of sample was injected.

(V) THE MOBILE PHASE

The mobile phase composition at first glance appears quite simple but under close examination is shown to consist

of a total of four solvents. The chloroform used to prepare the mobile phase contains 1 per cent ethanol as a preservative. When 80 milliliter of this chloroform was mixed with 20 milliliter of hexane and then saturated in a ratio of 1:1 with distilled water, a total of 4 solvents are present in the resulting mobile phase. Chloroform, hexane, ethanol and water indecreasing order of concentration were found in the mobile phase.

To be sure of the amounts of ethanol and water present, and also that the mobile phase could be produced reproducibly an analysis of the mobile phase was undertaken. The analysis was carried out on a F and M Scientific/Hewlett-Packard Model 720 Gas Chromatograph (Niagara College instrument) equipped with a thermal conductivity detector. The actual operating parameters of the analysis are given below:

Column packing:	Porapak Type Q, 80/100 mesh
Carrier gas:	Helium
Flow rate:	60 milliliter/min.
Column temperature:	220^{o}C
Injector temperature:	180^{o}C
Detector temperature:	230^{o}C

Standard solution of ethanol and water in chloroform were prepared using chloroform with no ethanol preservative. At high concentrations of water in chloroform, ethanol (a known amount) was added to help solubilize the water.

RESULTS AND DISCUSSION

(I) The Mobile Phase

To ensure that the mobile phase could be prepared reproducibly, it was analyzed using a gas chromatograph with a thermal conductivity detector. In Figures 6A and 6B are chromatograms of typical ethanol and water spiked chloroform standards. Figure 6 shows a 80 microliter ethanol in chloroform standard and Figure 6B is an ethanol peak which represents 250 microliter which is necessary to solubilize the 80 microliter of water. The analysis of the mobile phase showed that 70 microliter of the water and 80 microliter of ethanol were present per 100 milliliter of mobile phase. The reproducibility results are summarized in Table 1 which shows that the mobile phase is very reproducible with small deviations. The typical chromatograms for a mobile phase shaken for 2 hours with water is shown in Figure 7A. Figure 7B shows the chromatogram obtained for a mobile phase composition prepared minutes before it was injected. Figures 7A and 7B illustrates that about 5 minutes of shaking is all that is necessary to water saturate the mobile phase.

(II) LINEARITY

Linearity curves for both benomyl (in the presence of 10,000 microgram per milliliter BIC) and MBC-n-PIC (in the presence of 10,000 microgram per milliliter PIC) were prepared using the peak height method. Standard solutions of 0.1 to 250 microgram per milliliter of benomyl and 0.5 to 1,000

Figure 6

Chromatograms of the Ethanol and Water standards used for the Analysis of the Mobile Phase

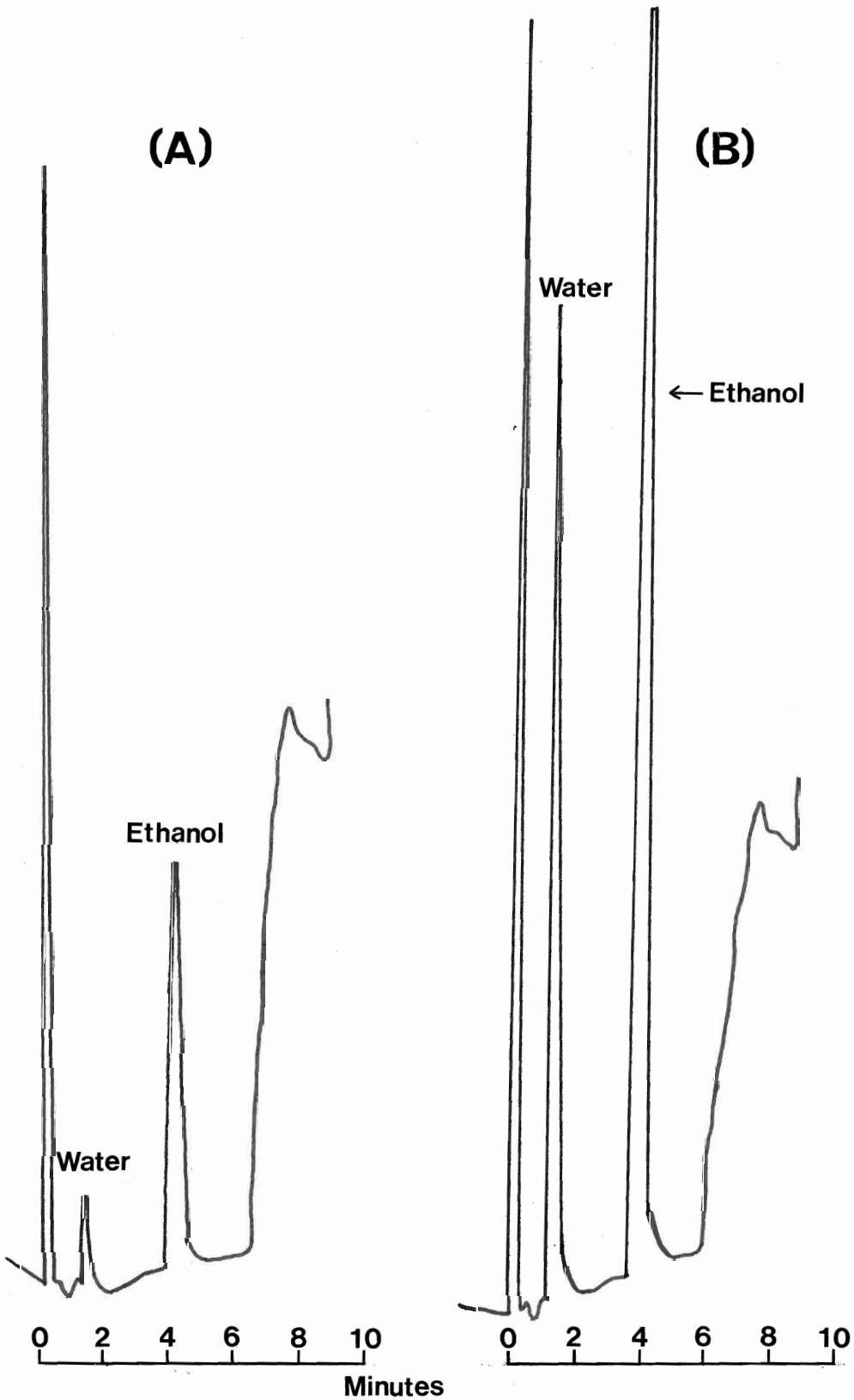


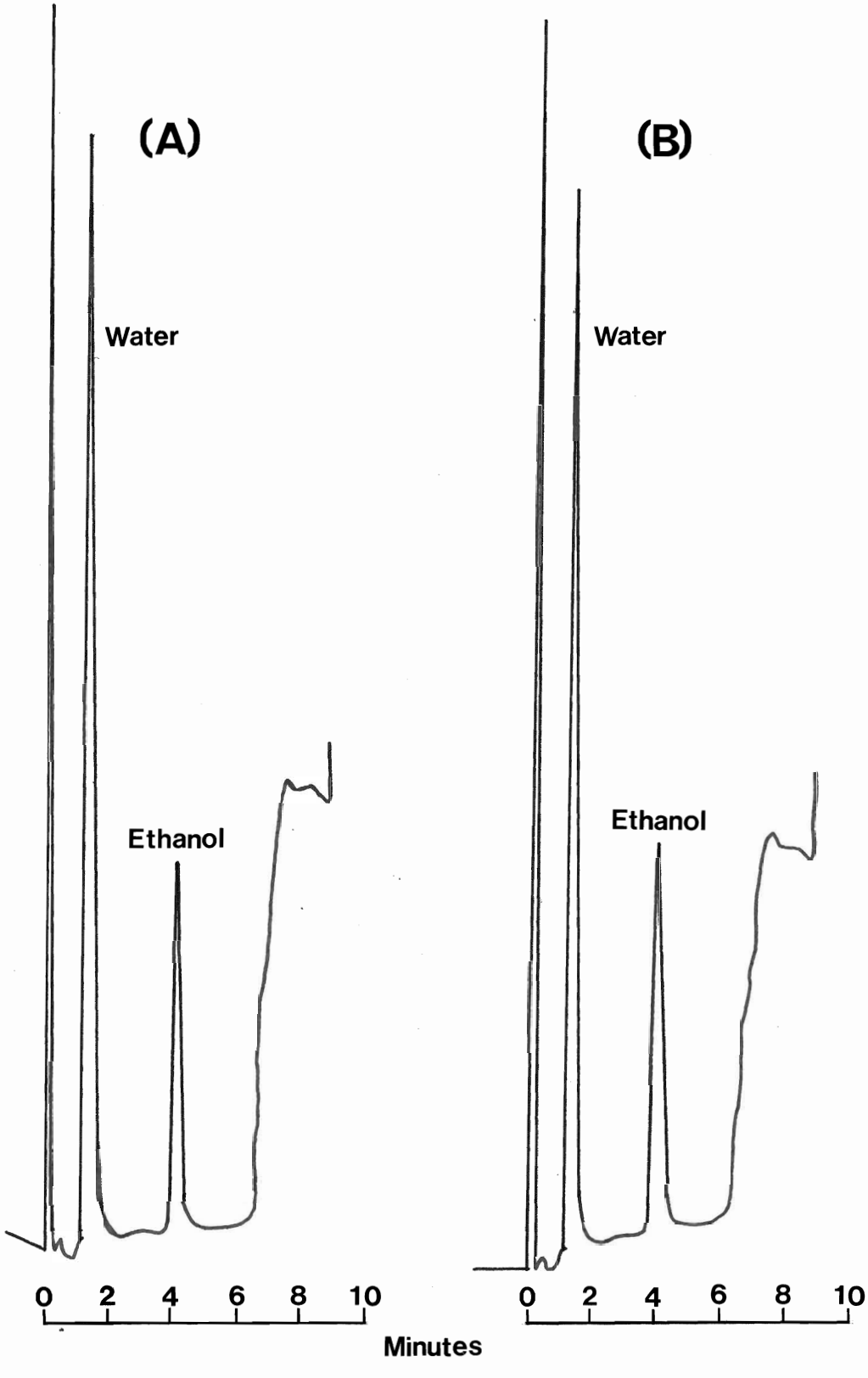
Table 1

The Water and Ethanol content of 5 different preparations of the Mobile Phase

Preparation Number	Water content (microliter / 100ml)	Ethanol content (microliter/100 ml)
1	70.2	81.3
2	70.2	81.3
3	70.7	80.0
4	70.2	77.5
5	70.7	81.3
Mean	70.4	80.3
Standard Deviation	0.27	1.65

Figure 7

Actual Chromatogram of the Mobile Phase Analysis



microgram per milliliter for MBC-n-PIC were run with the results shown in Tables 2 and 3 respectively. The actual working range for benomyl and MBC-n-PIC has been plotted in Figures 8 and 9 respectively. Both benomyl and MBC-n-PIC are linear in this working range. The linearity plots shown that both compounds follow the Beer-Lambert law that is A=EC where A= absorbance, E= extinction coefficient and C= concentration expressed in microgram per milliliter. Equation A=EC assumes a pathlength of 1 cm.

Table 2

Linearity Results for Benomyl

Sample Concentration microgram/ml	Detector Scale A.U.F.S.	Peak Height (mm)	Peak height calculated at Detector scale 0.0025
0.00	0.0025	0	0
0.10	0.0025	24	24
0.25	0.0025	50	50
0.50	0.0025	94	94
1.0	0.0025	184	184
2.5	0.01	124	496
5.0	0.02	128	1024
10.0	0.04	126	2016
25.0	0.08	156	4992
50.0	0.16	144	9216
100.0	0.32	144	18432
250.0	1.28	88	45046

Table 3

Linearity Results for MBC-n-PIC

Sample Concentration microgram/ml	Detector Scale A.U.F.S.	Peak Height (mm)	Peak height calculated at Detector scale 0.0025
0.0	0.0025	0	0
0.5	0.0025	26	26
1.0	0.0025	58	58
5.0	0.005	155	310
10.0	0.01	153	612
25.0	0.02	202	1616
50.0	0.04	208	3328
100.0	0.08	208	6656
250.0	0.32	126	16128
500.0	0.64	131	33536
1000.0	1.28	126	64512

Figure 8

Plot of Linearity for Benomyl

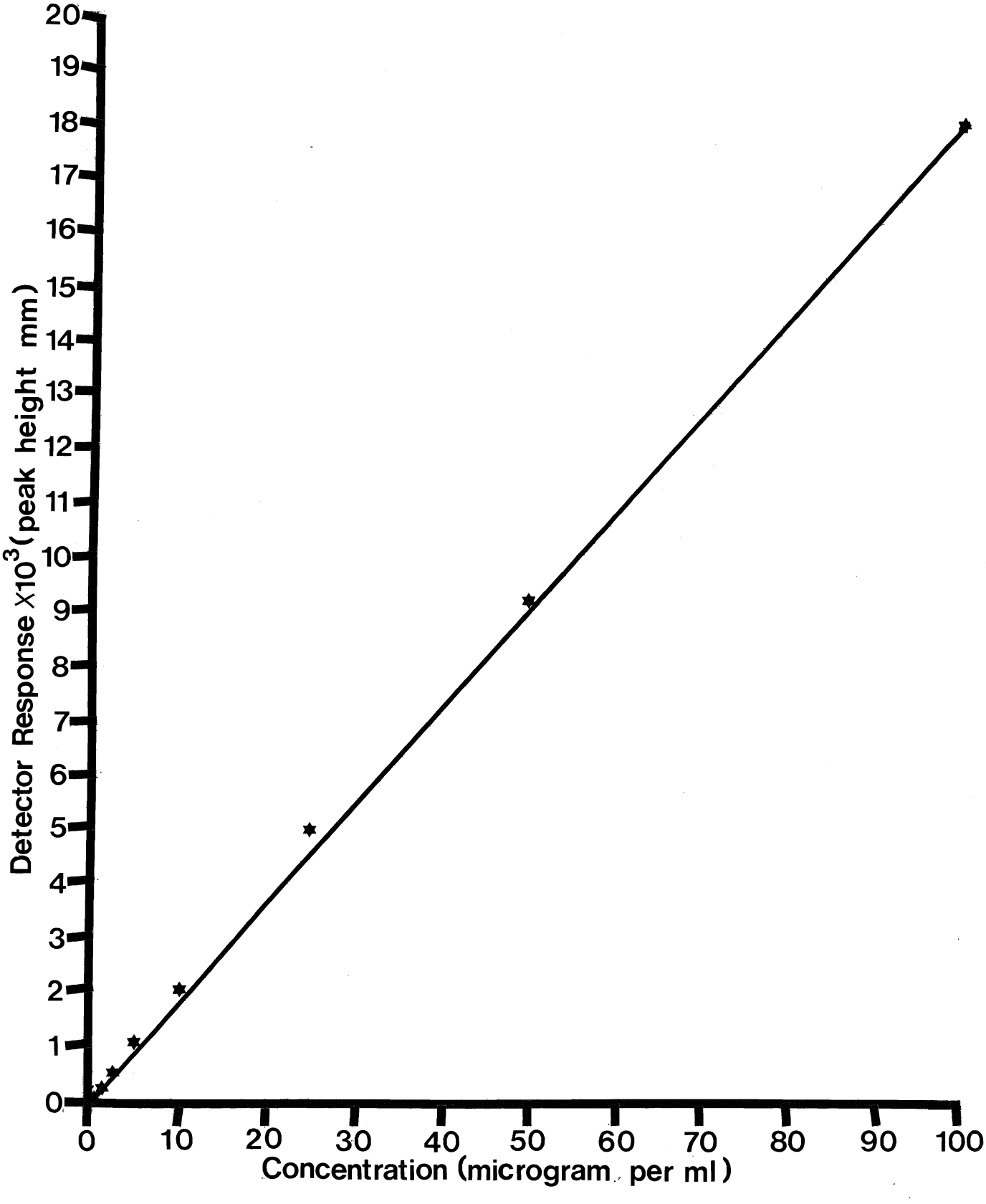
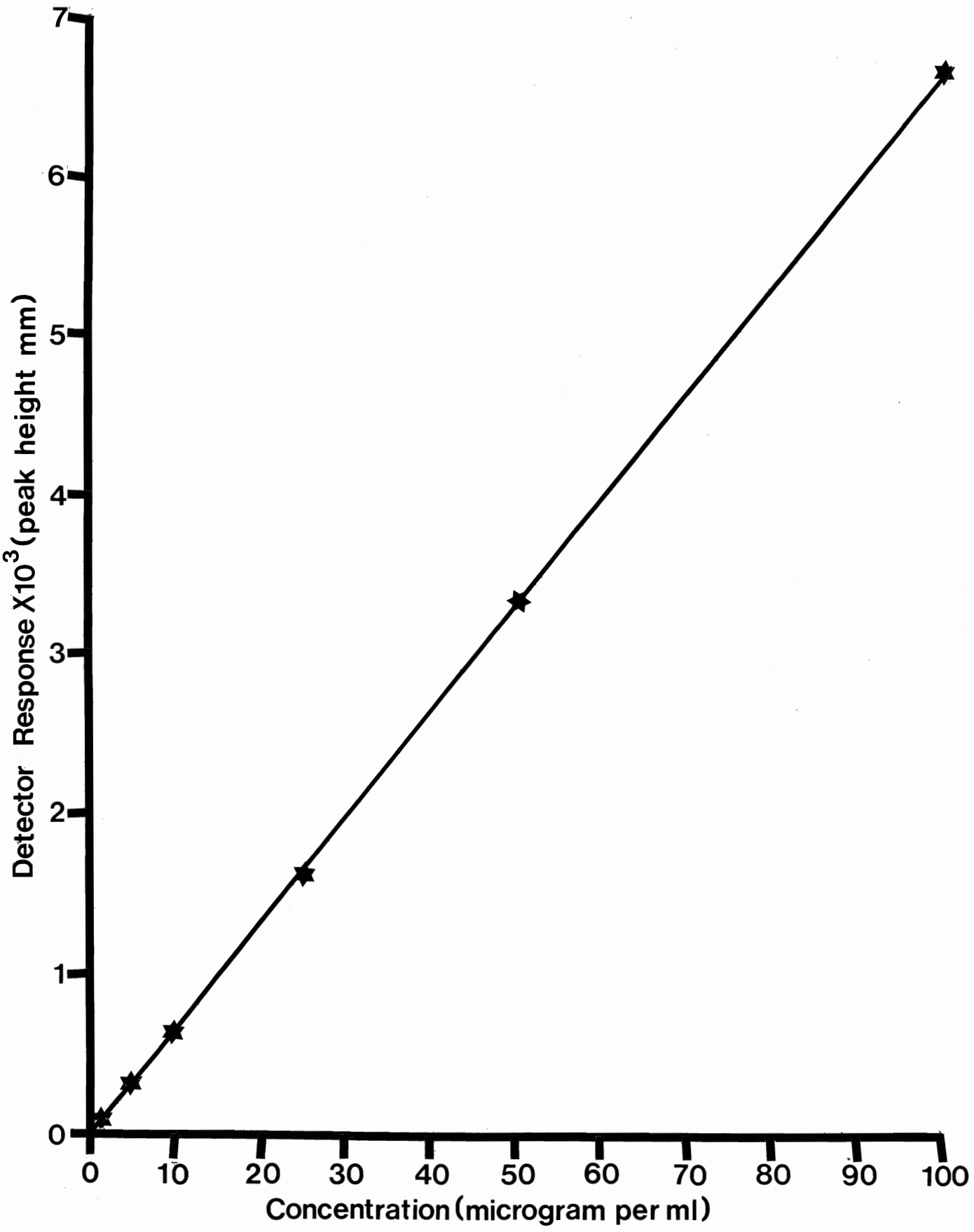


Figure 9

Plot of Linearity for MBC-n-PIC



(II) RECOVERY STUDY

Two separate spiking suspensions were prepared, one for benomyl and another for MBC. A benomyl suspension was prepared from Benlate 50 per cent wettable powder which was prepared at a rate yielding 500 microgram per milliliter active ingredient. The wettable powder must be analyzed using the low temperature solubilization technique method developed by Chiba (4). Once the per cent intact benomyl has been determined the weight of wettable powder weighed out will yield a final concentration of benomyl in water of 500 microgram per milliliter. A total of 5 milliliter and 1 milliliter of this suspension which contains 2,500 microgram and 500 microgram of active ingredient respectively, were added to, two 50.0 gram leaf samples. The leaf sample was tumbled in a Mason jar for a period of 10 minutes to distribute the wettable powder evenly on the leaves. A known weight of the wettable powder is taken and placed in an oven at 120^{o}C for twenty-four hours to convert the benomyl present completely to MBC. The conversion of benomyl to MBC was confirmed by analyzing a portion of wettable powder using the low temperature solubilization technique for the analysis of Benlate wettable powder (4). An aqueous suspension of MBC thus formed was prepared at a concentration of 250 microgram per milliliter. A total of 5 milliliter and 1 milliliter of the suspension which contains 1,250 and 250 microgram of MBC was added to 50.0 gram apple leaf samples.

Once again the suspension was added to the leaves and was tumbled for 10 minutes to evenly distribute the wettable powder suspension.

The extraction of benomyl and MBC simultaneously is very difficult for several reasons. First the extracting solvent must be a good solvent for both benomyl and MBC, such as p-dioxane, but at 12°C p-dioxane freezes. The extraction must take place at a low temperature, preferably 1°C to substantially slow down the decomposition of benomyl when it is being extracted. This does not rule out using p-dioxane but it would have to be small percentage of a solvent mixture in order to use it at 1°C. Secondly if chloroform is used as an extracting solvent the solubility is extremely poor for MBC. Thirdly the extracting solvent in order to solubilize MBC will have to include p-dioxane but the decomposition of benomyl is much faster in p-dioxane than chloroform, so the amount of p-dioxane included must be kept to a minimum.

The first extracting solvent that looked good was composed of 20 per cent p-dioxane, 64 per cent chloroform and 16 per cent hexane; with this mixture both benomyl and MBC could be solubilized and yet the p-dioxane concentration is small enough not to significantly contribute to benomyl' decomposition. Apple leaves were spiked with 1,200, 400, and 200 microgram which yielded recoveries for MBC of 71, 64 and 59 per cent respectively. This recovery of MBC was

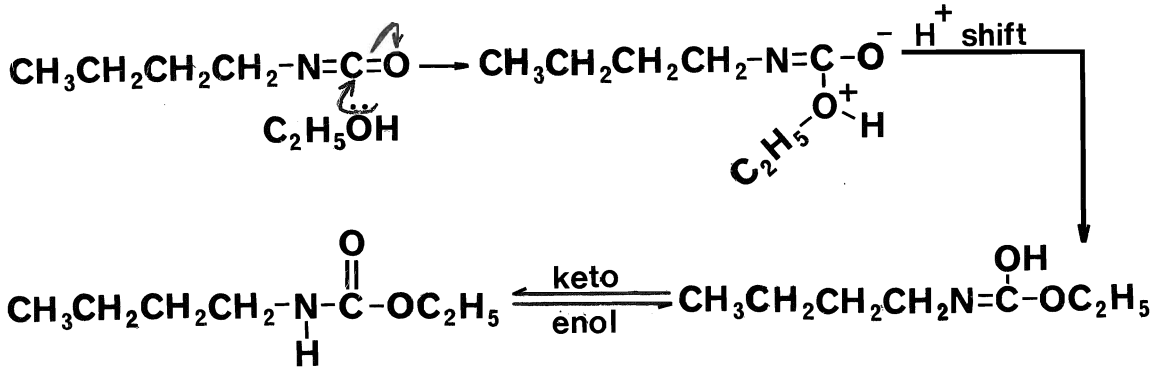
too low, as a goal of 80 per cent recovery for both benomyl and MBC was set. However this same extracting solvent was excellent for the extraction of benomyl. For the 4,200 and 2,100 microgram of benomyl spiked recoveries of 89 per cent respectively for both were obtained.

An improvement was required in the extraction procedure to make it an acceptable method. The efficiency of the MBC extraction was expected to be increased by freeze drying the leaves. To test this idea a 30 gram sample of Whatman filter paper was spiked with 1 milliliter of 1,328 microgram of wettable powder containg MBC . Using 5,000 microgram per milliliter of PIC in chloroform at 1°C a recovery of 79.3 per cent resulted. The second 30 gram filter paper sample was spiked with 1 milliliter of 1,328 microgram MBC and 20 milliliter of water. Using the same extraction procedure as above 71.6 per cent of the MBC was recovered; there are two reasons for this reduction, first the partitioning of MBC into chloroform was reduced in the presence of water and secondly PIC which enhances the extraction efficiency (to be covered later) decomposed rapidly in water, and therefore the effective concentration of PIC was reduced.

The extraction efficiency of MBC is also effectively increased by the addition of PIC to the chloroform extracting solvent. The addition of PIC increases the solubility of MBC by derivatizing MBC to MBC-n-PIC, a benomyl like

compound which is very soluble in chloroform. To illustrate the effect of PIC on MBC's extraction efficiency two 50 gram samples of leaves were spiked with 1,200 microgram of MBC each. Both leaf samples were freeze dried to remove the water present in the leaves. The leaf sample that was extracted with PIC had a recovery of 86.8 per cent while the leaf extract, extracted with no PIC had a recovery of 77.5 per cent. So using the PIC to derivatize the MBC to MBC-n-PIC increased the extraction efficiency of MBC by 9 per cent.

Two different types of chloroform are available, one with a 1 per cent $^{v}/_{v}$ ethanol preservative and another with no ethanol preservative. The chloroform used for extraction must be the one with no ethanol preservative. This is because ethanol will react with PIC and decompose it even when the extracting solvent is at $1^{o}C$. The decomposition of BIC by ethanol is shown below.



As pointed out previously the reduction of PIC present

in the extracting solvent will reduce the extraction efficiency of MBC. Satisfactory recoveries for benomyl and MBC were obtained by using 5,000 microgram per milliliter of PIC in chloroform (no ethanol preservative). A summary of the resulting recoveries for both benomyl and MBC are contained in Table 4.

Table 4

Summary of Benomyl and MBC Recovery

Sample Concentration per 50 gram of Leaf	Leaf Type	Sample	Benomyl Recovery	MBC Recovery	Mean and Standard Deviation
1200 microgram		MBC			
	Mac	1	-	81.3%	
	Mac	2	-	79.5%	
	Del	3	-	79.8%	
	Del	4	-	84.1%	81.2% ± 2.1
250 microgram		MBC			
	Del	1	-	78.2%	
	Del	2	-	77.3%	
	Del	3	-	77.0%	
	Mac	4	-	80.6%	
					78.3% ± 1.6
2500 microgram		Benomyl			
	Mac	1	86.2%	-	
	Mac	2	85.5%	-	
	Del	3	82.6%	-	
	Del	4	81.2%	-	
					83.9% ± 2.4
500 microgram		Benomyl			
	Mac	1	87.4%	-	
	Del	2	87.6%	-	
	Mac	3	84.6%	-	
	Mac	4	83.3%	-	
					85.7% ± 2.2

(IV) THE FORMATION OF THE MBC-n-PIC DERIVATIVE

It is extremely difficult to quanitatively extract both benomyl and MBC, and to simultaneously chromatograph them. The difference in polarity and solubility between benomyl and MBC is significant and makes the extraction of both very difficult. Because of these characteristics it is also very difficult to elute both compounds on a high performance column in a reasonable length of time under conditions suitable to separate both from the apple leaf extract background. By derivatizing the MBC and using PIC it is possible to extract both benomyl and MBC as MBC-n-PIC thereby eluting both in a reasonable length of time. In the extraction procedure the MBC must be converted to MBC-n-PIC and the rate of conversion must be faster than the rate at which benomyl degrades to MBC and BIC. If this was not the case the true concentration of benomyl could never be determined. The time required to form the MBC-n-PIC derivative at $1^{o}C$ in chloroform is about 11.5 min., as shown in Table 5. Since benomyl is stable at $1^{o}C$ for more than 1 hour, the MBC can be derivatized to MBC-n-PIC and simultaneously determined, with the presence of benomyl without fear of either degrading (Table 6).

Since benomyl and MBC-n-PIC are so similar it would be expected that MBC-n-PIC should exhibit similar kinetics. So when an excess of PIC is present in a solution containing MBC, the MBC should be quantitatively converted to MBC-n-PIC.

Table 5

The Formation of the MBC-n-PIC Derivative from MBC (75 microgram per ml) at 1°Celsius

Time after addition of n-PIC (2000 microgram per ml) min.	Peak height of MBC-n-PIC (mm)
2	122
11.5	219
25	224
38	218

Table 6

Stability of Benomyl in chloroform at 1° Celsius

Time (min)	Benomyl (peak ht. mm)
0	153
11	154
26	153
41	154
57	154

If this is true then by having an equal amount of both PIC and BIC in a solution containing MBC, then very similar rates of formation for both products would be expected. This is exactly what is seen in Table 7. This shows that the mechanism for the reformation for both benomyl and MBC-n-PIC is identical. When the peak height of the MBC-n-PIC derivative is measured against a standard of MBC-n-PIC, it was found that MBC can be quantitatively converted to MBC-n-PIC.

Table 7

The Formation of Benomyl and MBC-n-PIC from MBC in 2000 microgram/ml n-BIC and 2000 microgram/ml n-PIC at 1° Celsius

Time after addition of the two isocyanates (min)	Benomyl peak height (mm)	MBC-n-PIC peak height (mm)
1	37	38
4	74	84
9	92	92
22	96	95
27	95	95
40	103	106

(V) STABILITY OF BENOMYL AND THE MBC-n-PIC DERIVATIVE

The stability of benomyl in chloroform was studied with and without the presence of BIC. The stabilized benomyl was prepared by dissolving the weighed out benomyl in chloroform containing 5,000 microgram per milliliter BIC. The stability of the MBC-n-PIC derivative with and without PIC was also studied. The MBC-n-PIC was dissolved into chloroform containing 5,000 microgram per milliliter of PIC, to stabilize the MBC-n-PIC.

For benomyl in chloroform (no ethanol preservative) at $1^{o}C$ no decomposition of benomyl was observed for a minimum period of 57 minutes. The stability was determined by continuous monitoring of the benomyl peak height on the HPLC versus time. The results obtained are shown in Table 6. When benomyl was dissolved into 5,000 microgram per milliliter of PIC in chloroform (no ethanol preservative) at $1^{o}C$ no conversion of benomyl to the MBC-n-PIC derivative was observed for a minimum of 65 minutes. The results obtained are shown in Table 8. The study of benomyl in PIC containing chloroform was necessary to be sure no benomyl was being converted to the PIC derivative. This was thought possible because benomyl conversion to MBC and BIC is reversible and if a high concentration of another isocyanate is present then it may be possible that benomyl would be converted to the PIC derivative. The result of this conversion if it were to occur would be to give erroneous results

Table 8

Stability of Benomyl in chloroform containing 5000 microgram per ml n-PIC at 1°Celsius

Time (min)	Benomyl (peak ht. mm)
6	154
19	153
33	152
48	153
65	154

for the amount of benomyl present because the benomyl would be slowly converted to the MBC-n-PIC derivative. However no MBC-n-PIC derivative was observed during the 83 minutes that benomyl was measured.

The same stability study for the MBC-n-PIC derivative was also undertaken. The MBC-n-PIC derivative showed no decomposition in chloroform at 1°C for a minimum period of 65 minutes, as shown in Table 9. The stability of MBC-n-PIC in 5,000 microgram per milliliter of BIC containing chloroform was also very good showing no coversion of the MBC-n-PIC derivative for a minimum period of 68 minutes. The results obtained are shown in Table 10.

The stability of both benomyl and the MBC-n-PIC derivative was investigated in the presence of 10 microgram per milliliter of hydrochloric acid. Benomyl at a concentration of 10 microgram per milliliter was prepared in chloroform (no ethanol preservative) in the presence of 5,000 microgram per milliliter of BIC. In addition hydrochloric acid was also added to the chloroform to yield a final concentration of HCl of 10 microgram per milliliter. The resulting solution was immediately scanned from 235 to 315 nm and was again scanned one hour later. The first scan is shown in Figure 10 and the scan obtained 1 hour later in Figure 11. No measurable difference in peak height or λ max was observed. The same procedure was also carried out for the MBC-n-PIC derivative, all concentrations and methods of preparation

Table 9

Stability of MBC-n-PIC in chloroform at 1° Celsius

Time (min)	MBC-n-PIC (peak ht. mm)
0	151
13	153
26	155
39	153
53	155
65	153

Table 10

Stability of MBC-n-PIC in chloroform containing 5000 microgram per ml n-BIC at 1° Celsius

Time (min)	MBC-n-PIC (peak ht. mm)
3	153
16	150
29	148
42	147
56	152
68	155

Figure 10
Scan of HCl spiked Benomyl

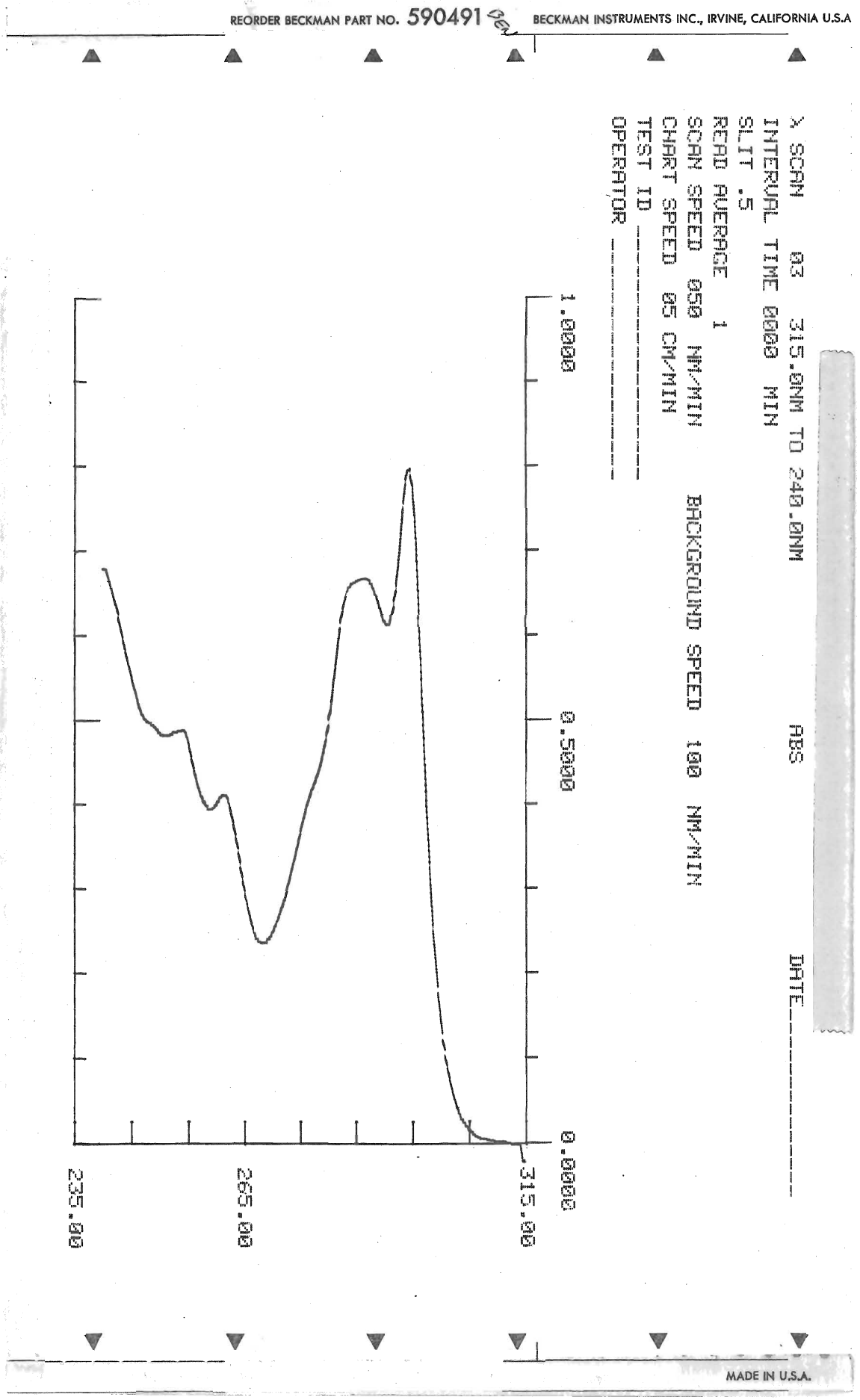
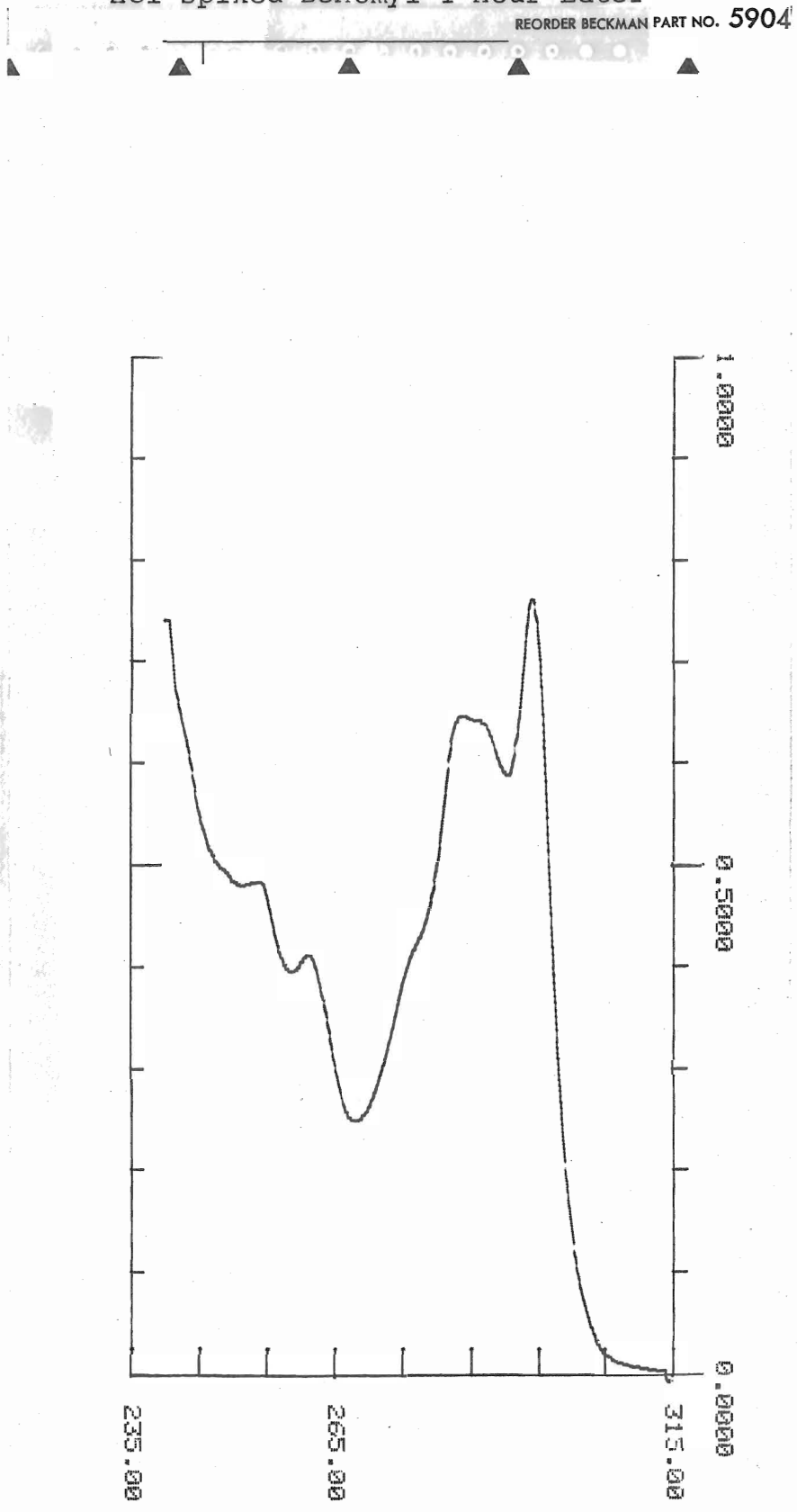


Figure 11
HCl spiked Benomyl 1 Hour Later



were the same as for benomyl. The first scan of MBC-n-PIC immediately after preparation is shown in Figure 12 and the spectra obtained 1 hour later shown in Figure 13. Again as for benomyl no measurable peak height difference or change in max was observed. The study indicates that no measurable amounts of degradation occurred in the presence of trace amounts of hydrochloric acid.

Many other isocyanates are available to derivatize MBC including methyl, ethyl and hexyl isocyanates. These other isocyanates may be very useful in moving the MBC derivative peak when interference from the extracted background occurs. The rate of formation of the methyl (MBC-MIC), ethyl (MBC-EIC) and hexyl (MBC-HIC) derivatives are in increasing order of hexyl, ethyl and methyl. The rate of formation being the fastest for the methyl and the slowest for hexyl. The rates of formation of the derivatives is related to the size of the respective isocyanate. The smaller the isocyanate molecule the faster it can react to form the derivative. When the column being used for analysis has deteriorated to a point where the benomyl and MBC-n-PIC peaks are no longer clearly separated, it is possible to use another isocyanate. For example when benomyl and MBC-n-PIC are no longer separated to the analyst's satisfaction it is possible to substitute the ethyl isocyanate (provided no interference occurs due to the background) and use this isocyanate to derivatize the

Figure 12
Scan of HCl spiked MBC-n-PIC

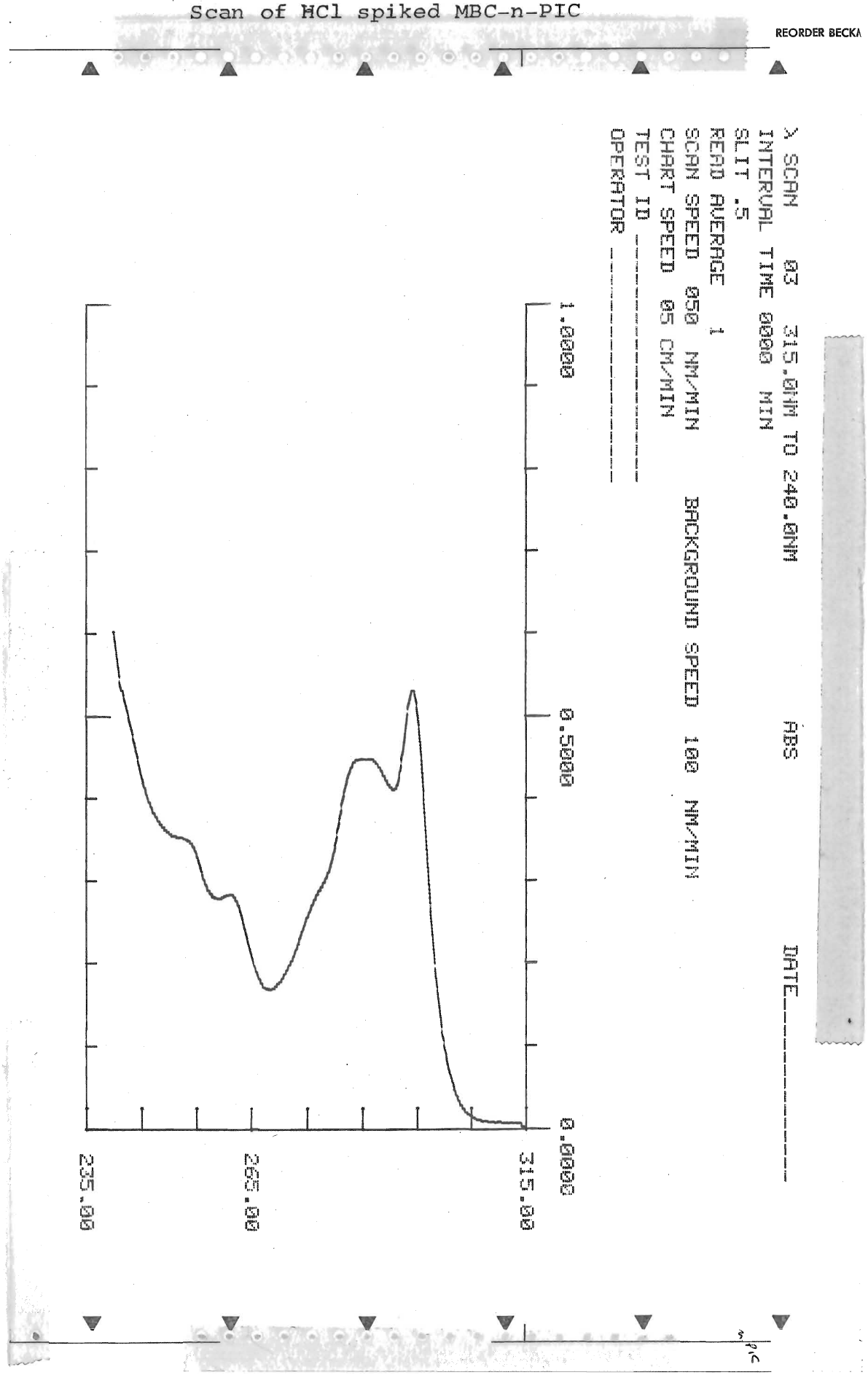
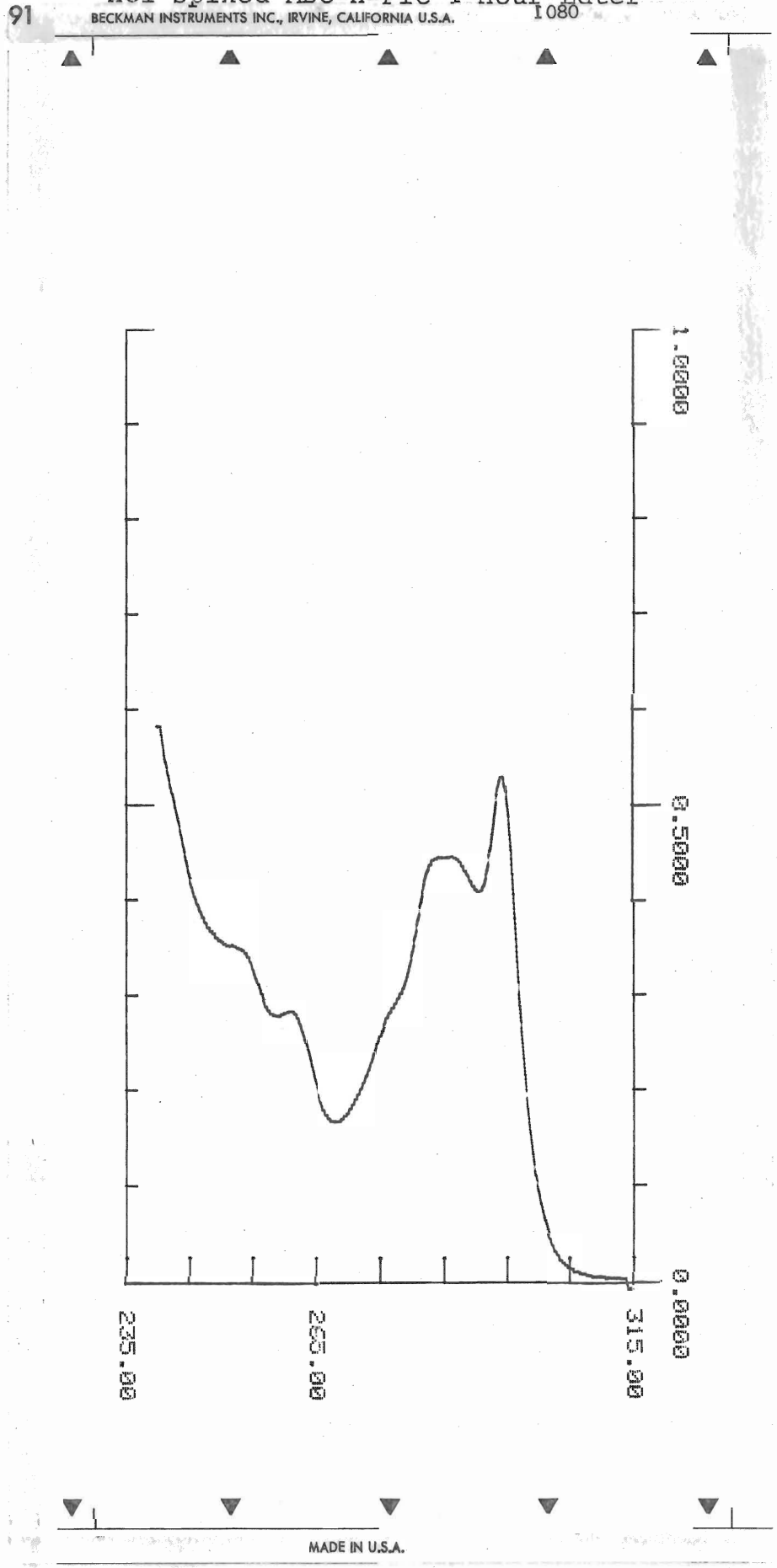


Figure 13

HCl spiked MBC-n-PIC 1 Hour Later



MBC. This is an important factor because as more and more injections are made onto the column it is constantly deteriorating, due to irreversible adsorption of extracted components. The typical elution pattern for MBC-HIC, MBC-n-BIC (benomyl), MBC-n-EIC, and MBC-MIC are shown in Figure 14. The different MBC isocyanate derivatives retention times relative to benomyl are given in Table 11.

Figure 14

Chromatogram of the Different Isocyanate Derivatives

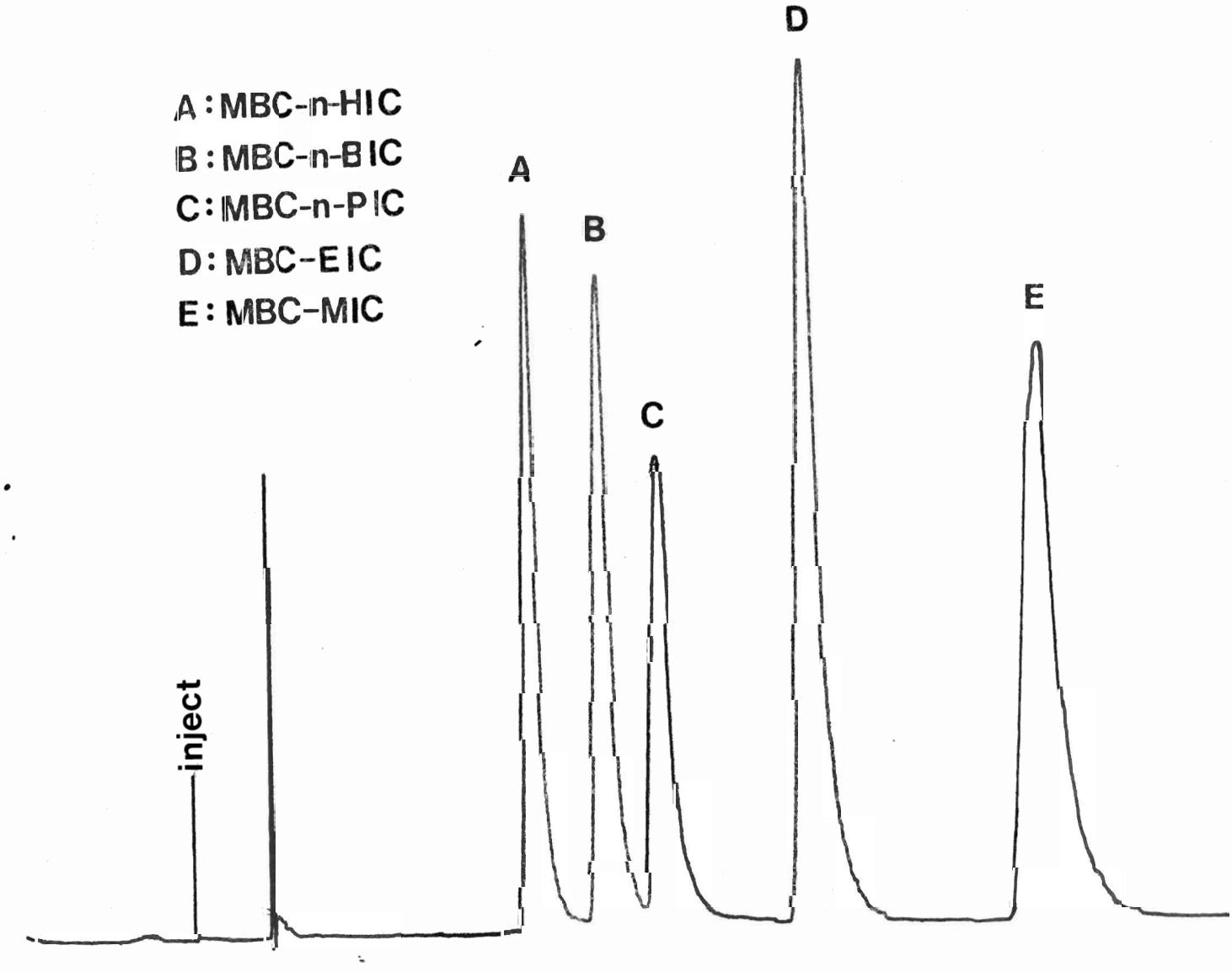


Table 11

Retention times relative to Benomyl of MBC Derivatives with different kinds of Isocyanates

MBC derivatives	Retention time relative to benomyl
MBC-n-Hexyl	0.81
MBC-n-Butyl (benomyl)	1.00
MBC-n-Propyl	1.15
MBC-Ethyl	1.55
MBC-Methyl	2.21

(VI) ELUTING PEAK IDENTIFICATION

The eluting sample peaks of benomyl and the MBC-n-PIC derivative were identified by retention time and a UV scan. The order of elution was always the benomyl peak first followed by the MBC-n-PIC derivative peak. This elution pattern is consistent with the character of a silica gel column. The more polar the compound the longer it is retained relative to less polar compounds. This can be shown from the structural difference between benomyl and MBC-n-PIC. Benomyl has a butyl isocyanate group while MBC-n-PIC has a propyl isocyanate group, the propyl being more polar than the butyl group and so the MBC-n-PIC elutes later. Identification was also attempted from a HPLC detector capable of scanning the peak in the flow cell. A Perkin-Elmer LC-55 detector was used for the scanning. Both benomyl and MBC-PIC were injected separately and as a mixture to check their respective retention times (Figure 15). Then benomyl and MBC-n-PIC were injected separately and each scanned. The spectra obtained for benomyl and MBC-n-PIC are shown in Figure 16 and 17 respectively. However the absorption characteristics of the two samples are sufficiently close to make them indistinguishable. The operating conditions for the sample elution along with the scan conditions are given below.

Flow rate: 3 milliliter/minute

Column: 5 micron 4.6 mm I.D. X 25 cm silica gel column (Brownlee)

Figure 15
Elution of Benomyl and MBC-n-PIC (scan run)

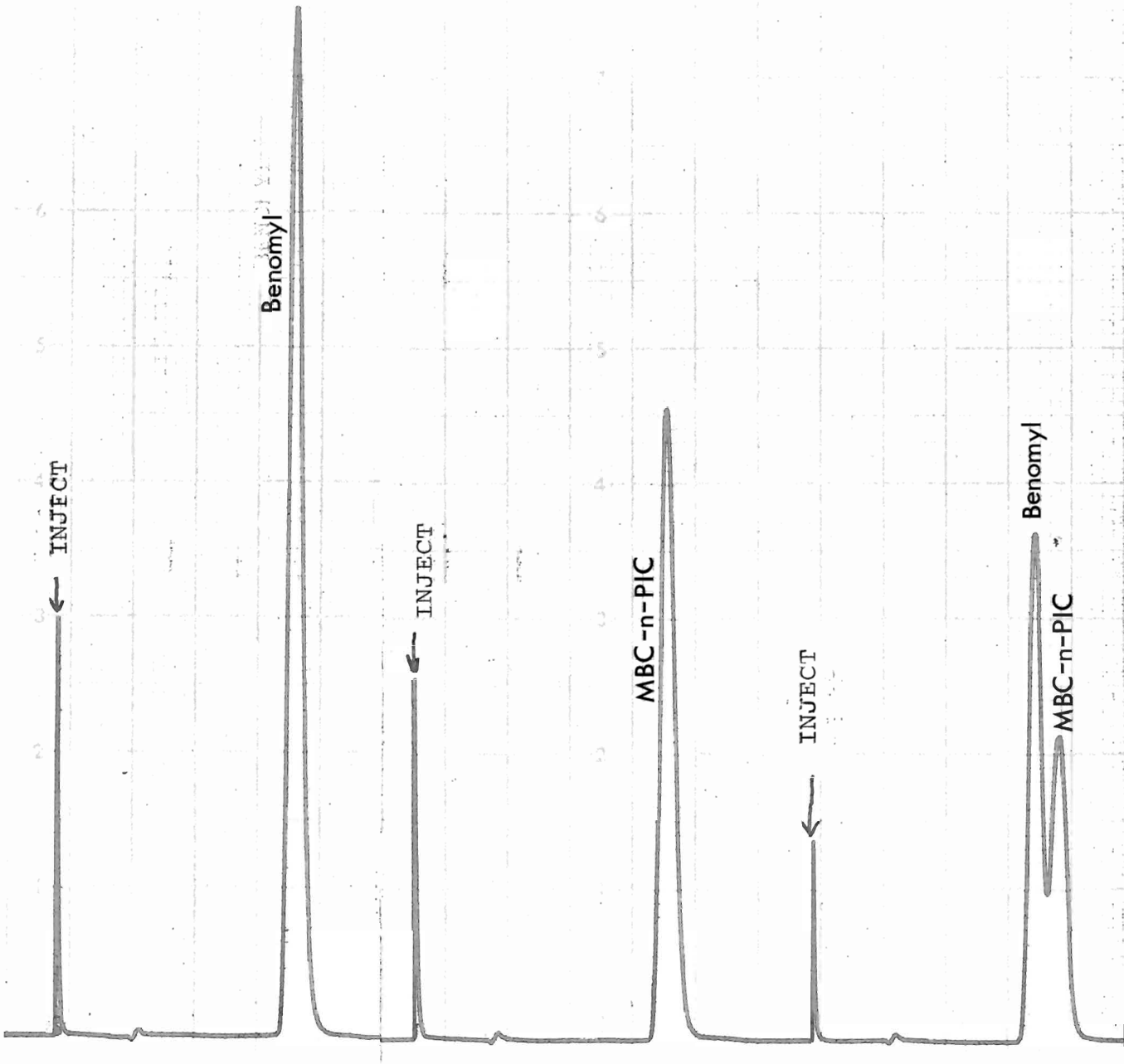


Figure 16
UV scan Benomyl using Perkin-Elmer LC-55

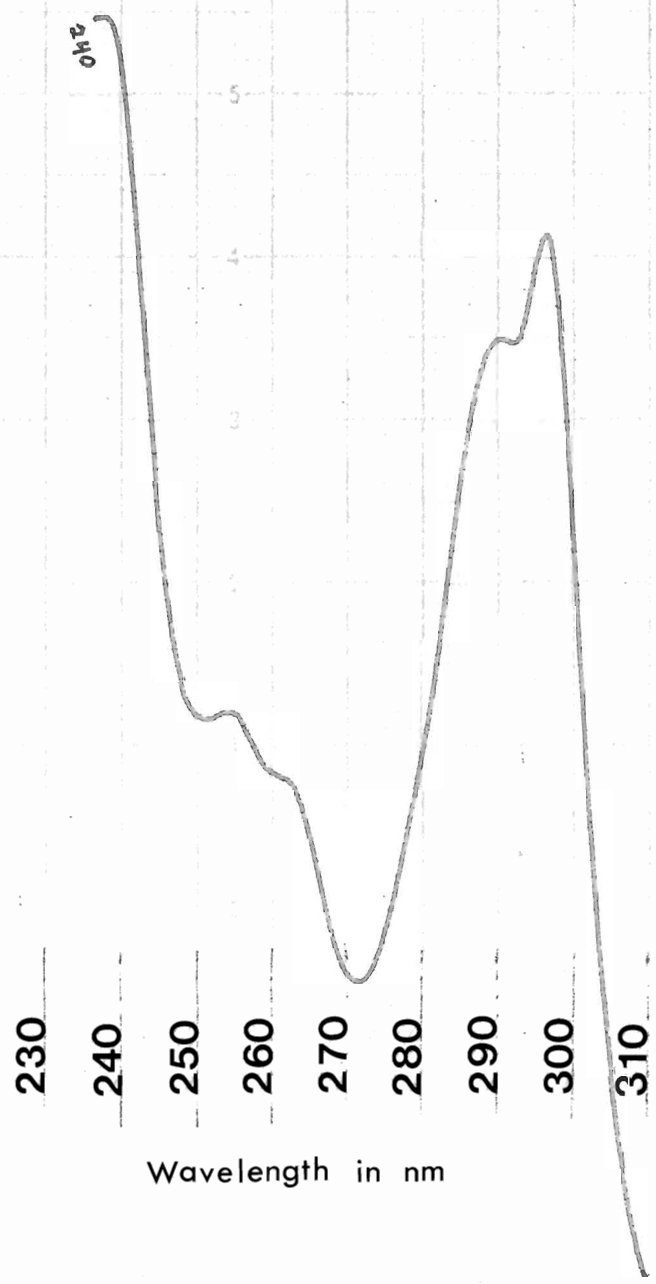
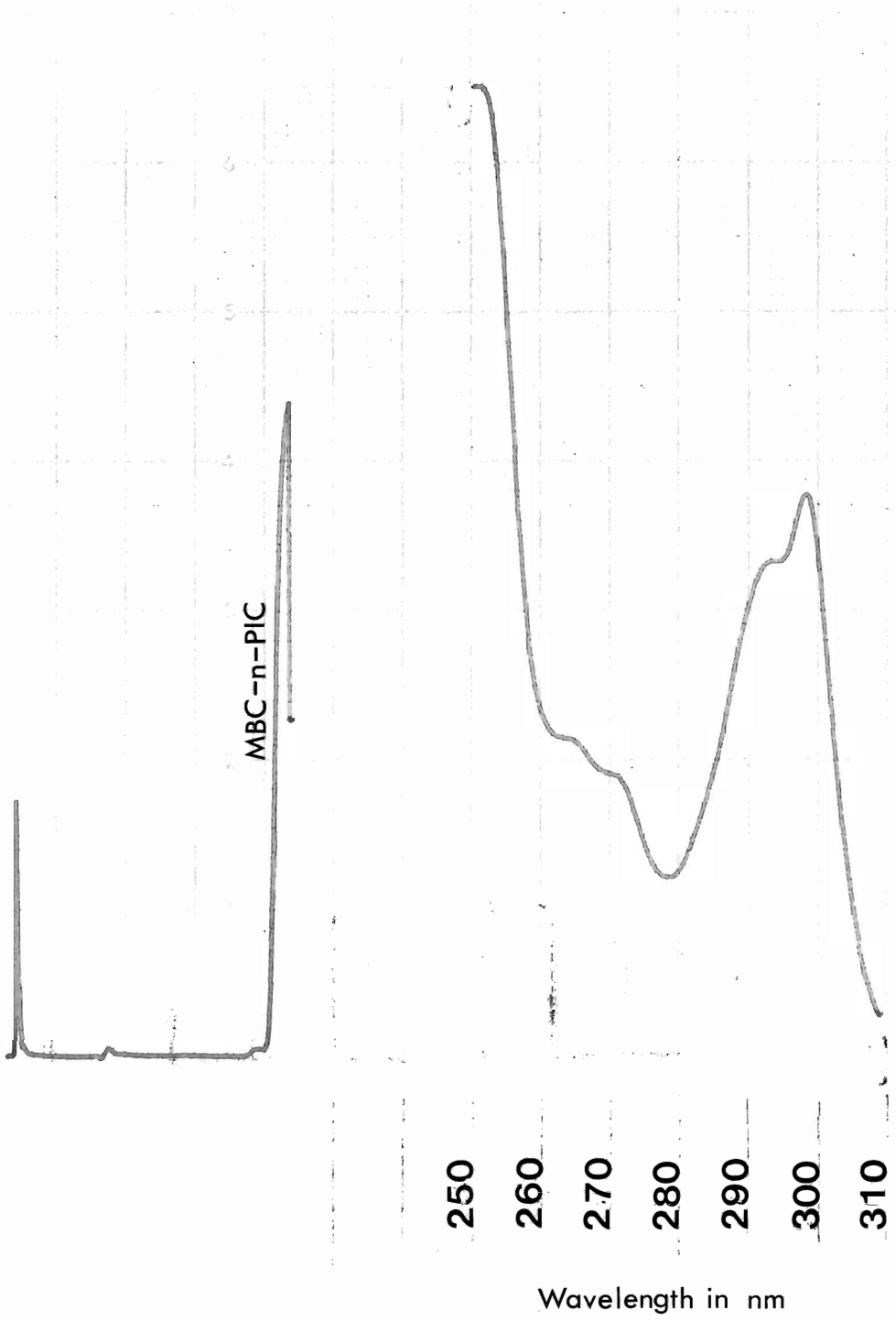


Figure 17
UV scan MBC-n-PIC using Perkin-Elmer LC-55



Mobile phase: Water saturated, 80 per cent chloroform, 20 per cent hexane

Chart speed chromatogram: 1 centimeter/minute

A.U.F.S. : 1.0

Response time: 2.0 second

Wavelength scan: 240 to 320 nanometer

Scan chart speed: 10 centimeter/minute

(XI) THE ANALYSIS OF BENOMYL AND ITS DEGRADATION COMPOUND ON APPLE FOLIAGE

The HPLC method developed was used to determine the rate of dissipation of benomyl and MBC after spray application on apple leaves. The Benlate 50.0 per cent wettable powder was applied at a rate of 1.68 kilogram/hectares to dwarf trees of cultivars McIntosh and Red Delicious. Two separate spray applications took place, one in 1977 and another in 1979. The 1977 spray application was a series of 3 sprays while the 1979 study consisted of one spray only. The samples collected for the 1977 study consisted of duplicate 50.0 gram samples and triplicate 50.0 gram samples for the 1979 study. Samples were taken immediately before and after a spray for all spray applications. After the leaves were collected the stems were removed and leaves stored in 1 quart Mason jars at -15 degree celsius till they were ready for analysis.

1977 STUDY

The HPLC analysis showed that the first application had a benomyl concentration of 115.7 microgram per gram of leaf. As time went on benomyl decreased due to evaporation, wash off due to rain and most importantly due to the degradation to MBC. The concentration of benomyl decreased to 32.7 microgram per gram which was 28.3 per cent of the oringinal deposit as shown in Figure 18 and Table 12. MBC was 15.2 microgram per gram after the initial spray and

Figure 18
Plot of 1977 Spray Application Results

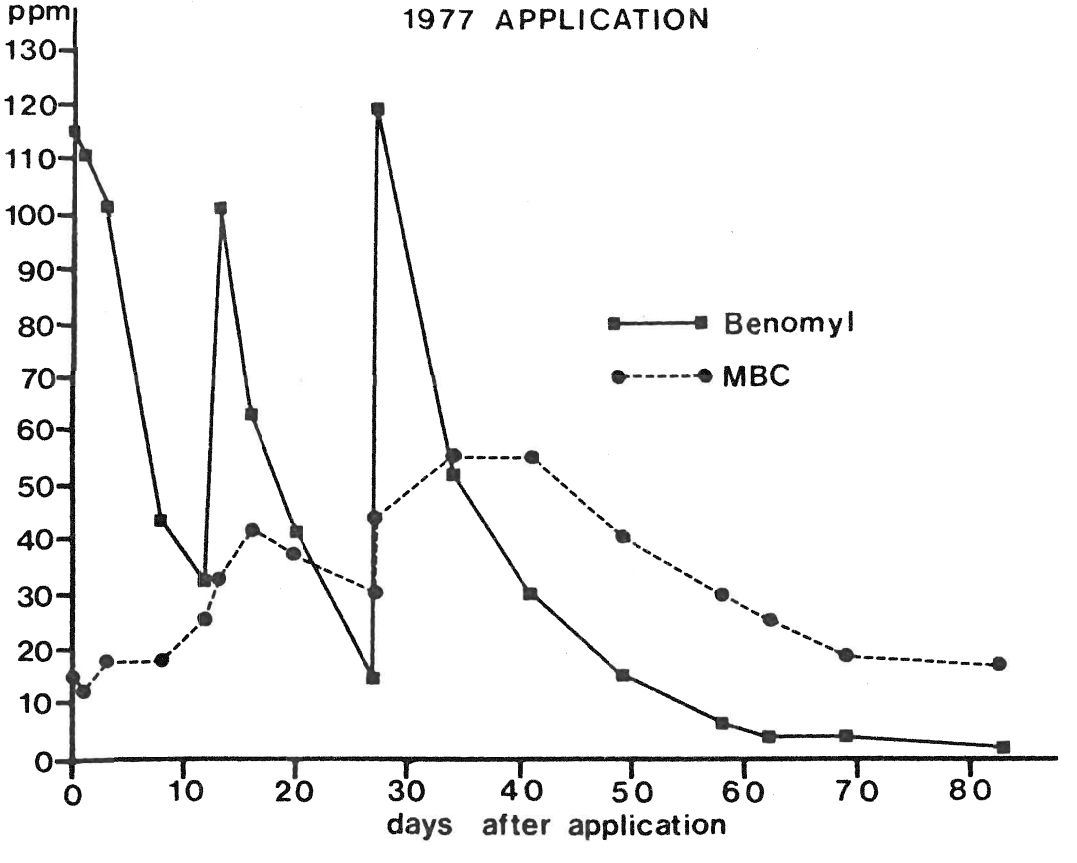


Table 12 Residues of Benomyl and MBC in Apple Foliage after Spray Applications in 1977 of Benlate® at Different Intervals

Date	Number of days after application		Benomyl				MBC			
	From first application	From each of successive applications	Average deposits, ppm	±	Standard deviation[a]	% in total residues	Average deposits, ppm	±	Standard deviation[b]	% in total residues
July 27	0		115.7	±	4.5	88.4	15.2	±	2.4	11.6
28	1		111.6	±	11.0	89.7	12.8	±	2.9	10.3
30	3		101.9	±	11.6	85.0	18.1	±	4.1	15.0
Aug. 4	8		43.9	±	5.3	69.9	18.8	±	2.0	30.1
8	12		32.7	±	2.3	55.9	25.8	±	2.9	44.1
9	13	0	101.8	±	5.9	75.5	33.1	±	3.6	24.5
12	16	3	63.0	±	7.2	60.4	41.5	±	7.9	39.6
16	20	7	41.5	±	8.1	52.1	38.0	±	6.8	47.9
23	27	14	15.2		4.0	33.0	30.8	±	5.5	67.0
23	27	0	119.4	±	11.2	73.2	43.7	±	7.0	26.8
30	34	7	52.1	±	7.0	48.2	55.8	±	4.7	51.8
Sept. 6	41	14	30.8	±	6.1	35.5	55.6	±	6.5	64.5
14	49	22	15.9	±	2.3	28.5	40.7	±	8.2	71.5
23	58	31	6.8	±	0.70	18.4	30.1	±	1.7	81.6
27	62	35	4.4	±	0.65	15.1	25.8	±	7.9	84.9
Oct. 4	69	42	4.2	±	1.9	18.7	19.1	±	4.3	81.3
18	83	56	2.6	±	0.96	14.5	17.1	±	7.6	85.5

[a] Average coefficient of variation is 16.0%.

[b] Average coefficient of variation is 18.2%.

and increased to 25.8 microgram per gram before the second application.

For the second application benomyl was 101.8 microgram per gram and decreased to 15.2 microgram per gram just before the last spray. The MBC concentration after the second spray was 41.5 microgram per gram just after the spray and decreased to 30.8 microgram per gram before the third spray.

The last application showed the benomyl concentration to be 119.4 microgram per gram and MBC was 43.7 microgram per gram. At the end of the 56 day period the benomyl concentration was 30.8 microgram per gram and MBC only 2.6 microgram per gram.

1979 STUDY

After the first and only spray application, the benomyl deposit was 88.9 microgram per gram which was lower than the initial deposit for the 1977 spray (Figure 19). The initial MBC concentration was lower for 1979, 3.7 microgram per gram as compared to 15.2 microgram per gram for 1977. The low MBC concentration was maintained throughout the season. Because only one spray was applied, MBC was unable to build up as had been the case in 1977. The highest concentration of MBC in 1979 was 13.7 microgram per gram as compared to a value of 55.8 microgram per gram in 1977, (Table 12, Table 13).

The rate of degradation on the two cultivars of apples was essentially the same in both years.

Figure 19
Plot of 1979 Spray Application Results

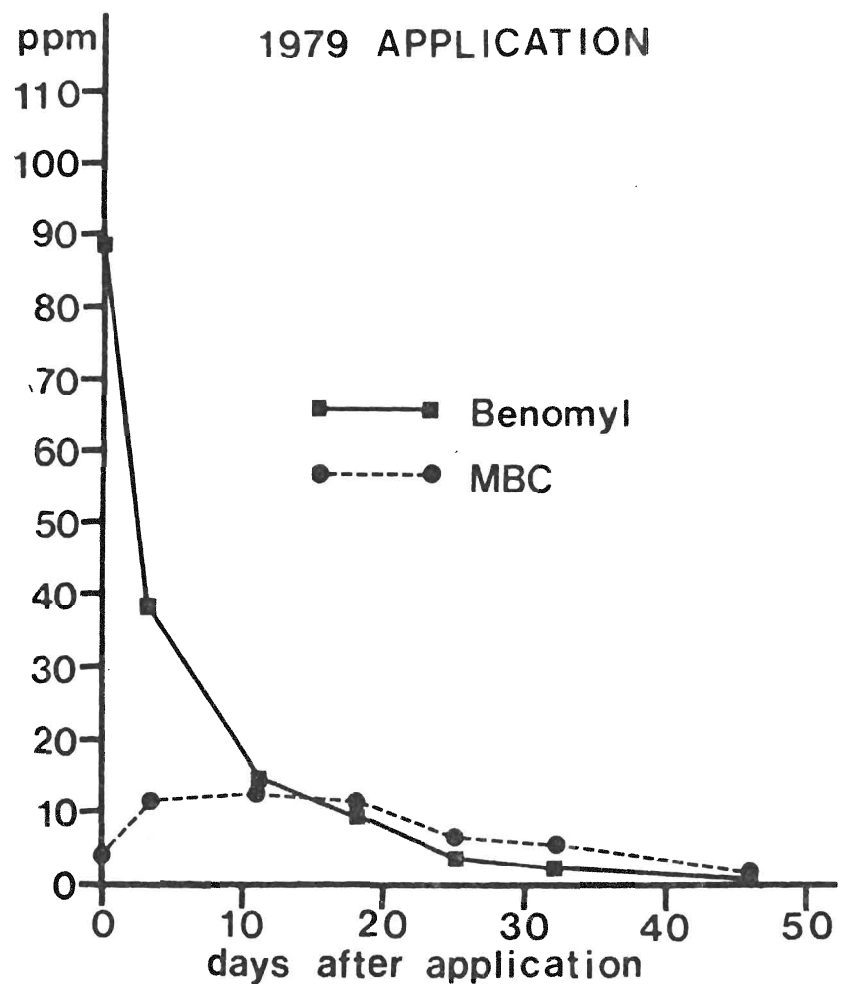


Table 13 Residues of Benomyl and MBC in Apple Foliage after Spray Application in 1979 of Benlate® at Different Intervals

Date		Number of days after application	Average deposits ppm	±	Standard deviation[a]	% in total residues	Average deposits ppm	±	Standard deviation[b]	% in total residues
Aug.	24	0	88.9	±	7.2	96.0	3.7	±	1.2	4.0
	27	3	38.8	±	2.3	76.8	11.7	±	1.1	23.2
Sept.	4	11	14.2	±	2.5	51.0	13.7	±	2.4	49.0
	11	18	9.5	±	0.60	46.2	11.4	±	2.7	53.8
	18	25	3.7	±	0.94	35.9	6.5	±	1.4	64.1
	25	32	2.7	±	0.35	33.3	5.3	±	0.70	66.7
Oct.	9	46	0.63	±	0.20	34.4	1.2	±	0.30	65.6

[a] Average coefficient of variation is 15.4%.

[b] Average coefficient of variation is 20.4%.

The interesting aspect of the results is that benomyl is rather stable on plant foliage. Benomyl was the dominant residue for the first 34 days after the application during July and August. It is during this time of year, July and August that protection is most critical. MBC was found during the entire sampling period and reached its highest concentration of 55.8 microgram per gram 7 days after the third application in 1977. It was at this time after the third spray that the MBC concentration was higher than that of benomyl and remained that way till the end of the study. The lowest concentration of MBC, 17.1 microgram per gram was reached at the end of the season (this excludes the first 3 days after the first spray) and was the lowest concentration during the entire 83 days as shown in Figure 18, Table 12.

(VIII) THE HPLC CHROMATOGRAM AND SENSITIVITY

The separation of benomyl from MBC-n-PIC and the separation of benomyl and MBC-n-PIC from the apple leaf extract was very good. A typical chromatogram of the control leaf extract is shown in Figure 20A, a mixture of 0.2 microgram per milliliter each of benomyl and MBC-n-PIC in Figure 20B, and a control leaf extract spiked with 0.2 microgram per milliliter each of benomyl and MBC-n-PIC in Figure 20C. The chromatograms in Figure 20 show that the area of elution of benomyl and MBC-n-PIC onthe control leaf extract is very clean and no interference is shown. Baseline resolution is achieved for benomyl and MBC-n-PIC. The majority of apple leaf extract components would be expected to be adsorbed onto the 3 cm pre-column used. Because no extra cleanup is necessary (which usually results in residue loss) the complete analysis only takes 20 minutes after extraction. No decomposition of benomyl during the HPLC run was observed which is due to two factors, first all preparation of the sample takes place at 1°C and secondly the presence of BIC in the extract minimizes the degradation of benomyl at room temperature.

The sensitivity possible with this method is very good. Figure 21 shows benomyl and the MBC-n-PIC derivative each at a concentration of 0.4 microgram per milliliter. The minimum detectable concentration would be 0.1 microgram per

Figure 20

Chromatograms of Apple Leaf Extract and Spiked Apple Leaf Extract

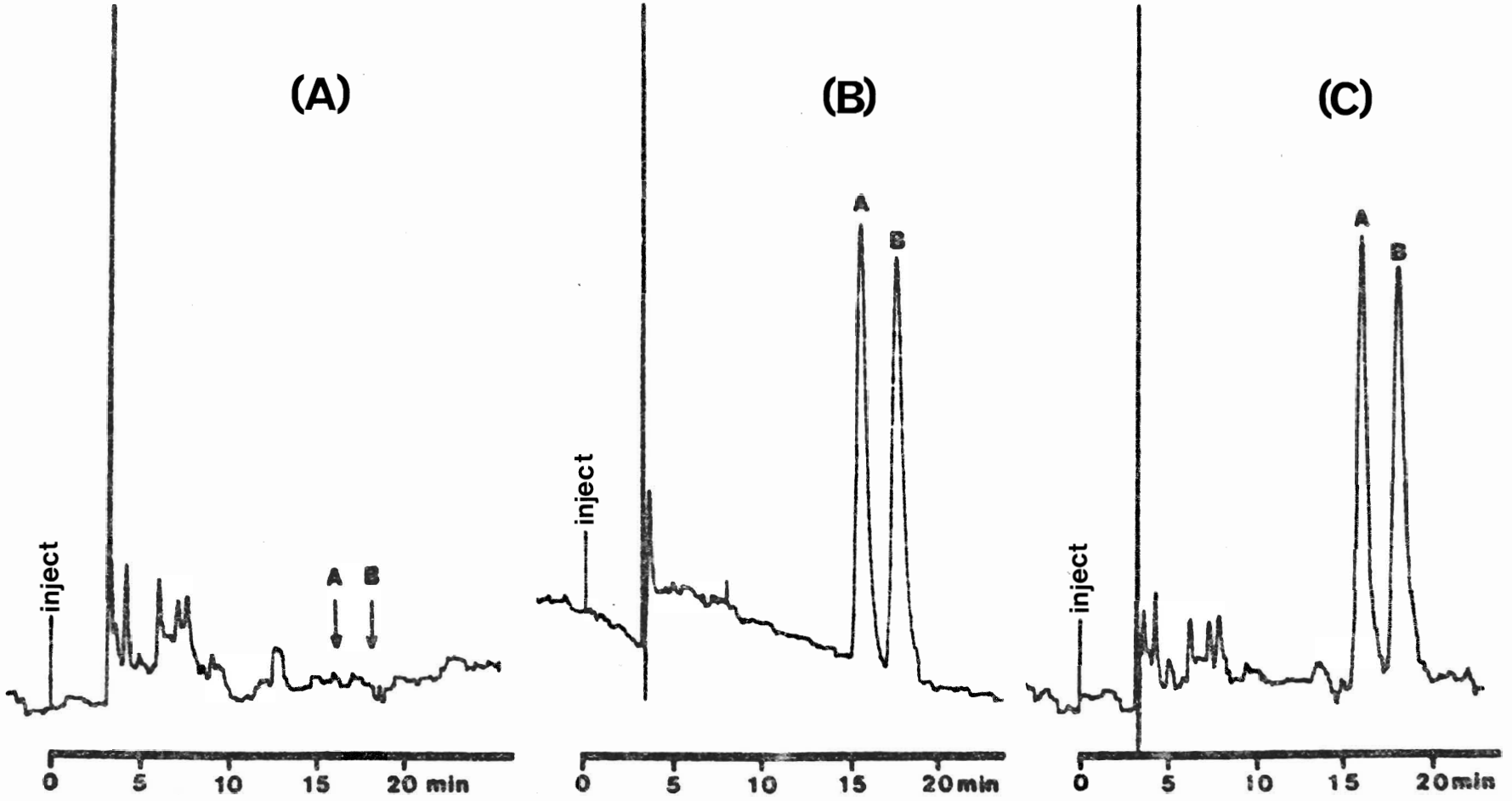
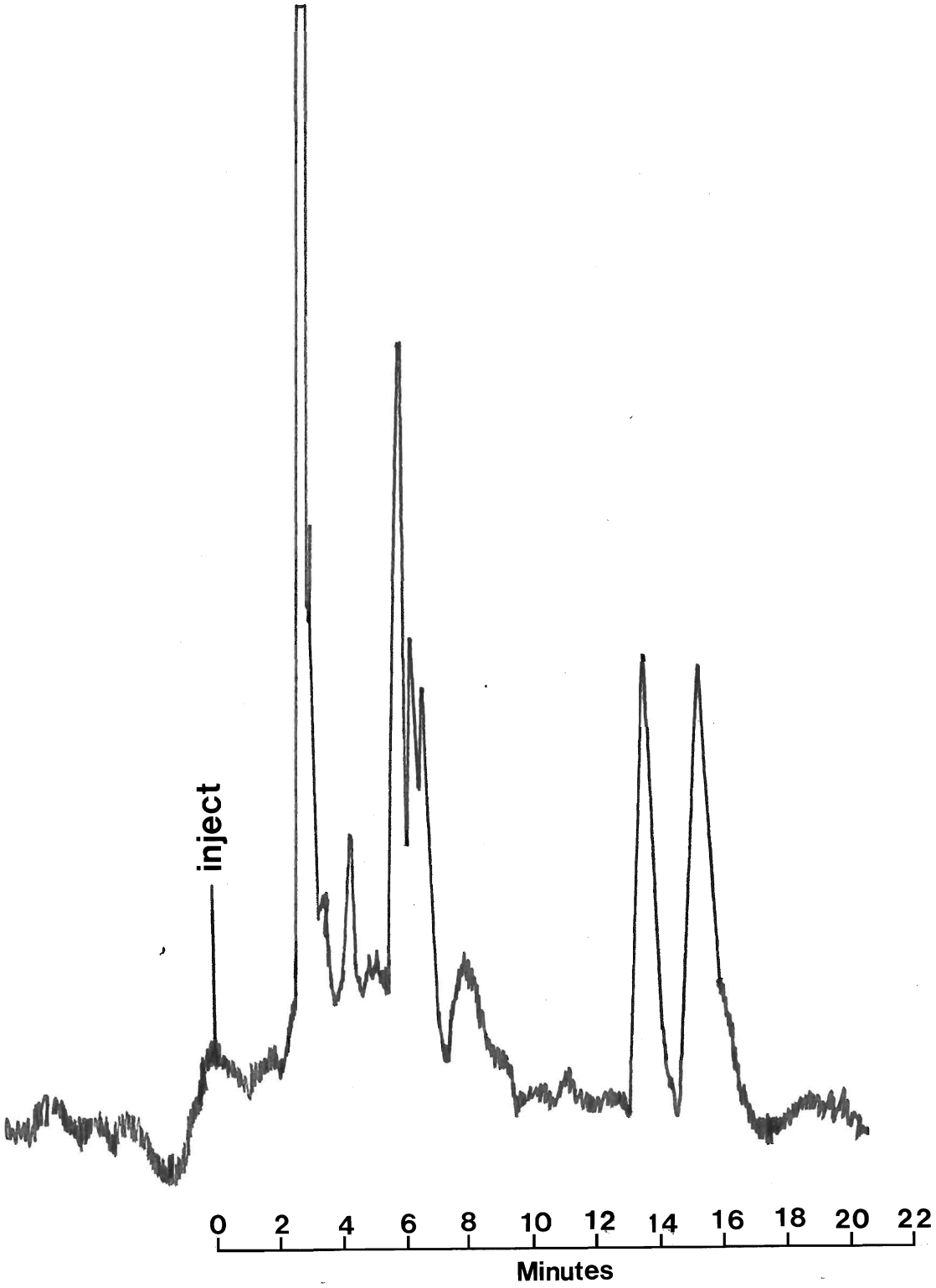


Figure 21

Sensitivity for Benomyl and MBC-n-PIC

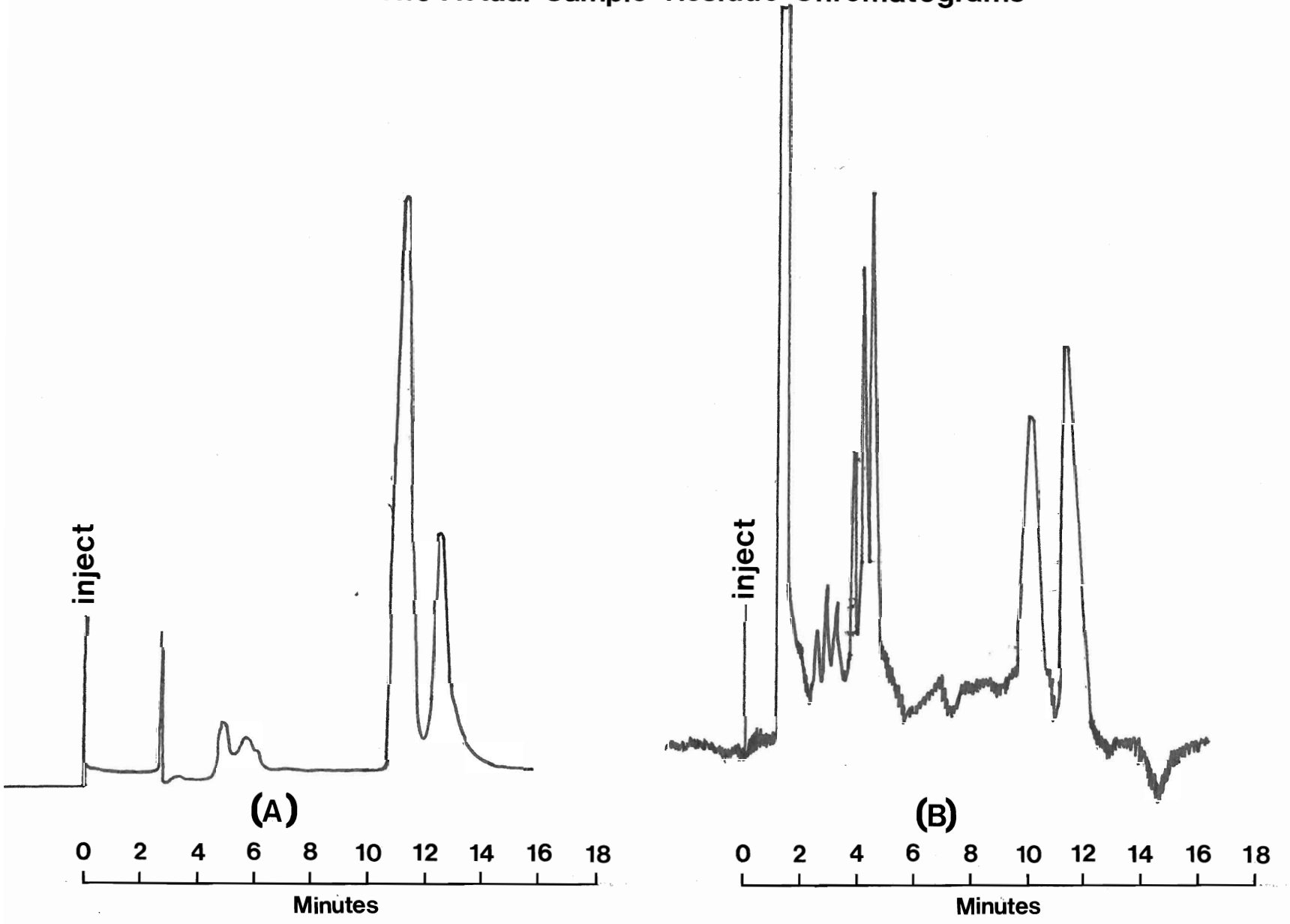


milliliter (conservatively) for both benomyl and MBC-n-PIC. The limiting factor here is that the lowest detector range scale was 0.0025. Since the noise at 0.0025 A.U.F.S. is still very good it is possible to amplify the signal at least twice and still obtain acceptable baseline noise. With this amplification it would be possible to increase the detectable level of benomyl and MBC-n-PIC to at least 0.1 microgram per milliliter. Another possibility for increasing the detection level is to use a larger sample loop. All the work done herein was accomplished using a 20 microliter sample loop. It is possible that a sample loop of greater size could be used without overloading the column thereby improving the minimum detectable level.

An actual chromatogram obtained of a sample taken right after spray application is shown in Figure 22. This chromatogram illustrates the very clean background at high concentrations of benomyl and MBC-n-PIC. Figure 22B illustrates the actual chromatogram of a low residue containing sample extract. Even at this low concentration of residues the area of elution for benomyl and MBC-n-PIC is very clean. The actual practical use of the method has been clearly demonstrated by the analysis of residues of benomyl and MBC on apple foliage.

Figure 22

Two Actual Sample Residue Chromatograms



CONCLUSIONS

The method described herein, is the first analytical method available for the simultaneous determination for both benomyl and MBC residues. This method needs no prior cleanup of the extract before HPLC injection, thereby avoiding the residue loss associated with cleanup and reducing the time required for analysis. The ability to derivatize MBC using other isocyanates provides flexibility in this method. By using different isocyanates it is possible to move the MBC peak around interfering background peaks. The isocyanates tried include hexyl (HIC), ethyl (EIC), and methyl (MIC). The chromatogram showing the elution of the different derivatives of MBC is shown in Figure 14. The relative retention times of the MBC derivatives to benomyl is shown in Table 11. The most important aspect of this method is the ability to do the analysis of benomyl present in samples, without fear of degradation due to solvent interactions.

The method was used to carry out analysis on treated apple leaves to study the persistance and degradation of benomyl. This kind of study was impossible before, because a capable analytical technique wasn't available.

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